Effect of Phosphorus Deficiency on Levels of Phosphorus Compounds in Spirodela

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Abstract. When Spirodela plants are transferred to a phosphate-deficient medium, growth slows down immediately, and ceases after 14 days. During this time, inorganic phosphate content falls from 30 to 0.7 μmoles/g fresh weight of tissue, phosphate ester content from 3.5 to 0.6 μmoles/g, phospholipid content from 3.5 to 1.2 μmoles/g, and residual phosphate (mainly RNA) content from 7.5 to 2.0 μmoles/g. Relative proportions of the various phosphate esters, and relative proportions of the various phospholipids, are not markedly affected by phosphate deficiency. Turnover rates of phosphate esters are somewhat higher in phosphate-deficient tissue. In control tissue, inorganic phosphate is present in 2 pools; a metabolic (12%) and a non-metabolic pool (88%). In phosphate-deficient tissues, most of the inorganic phosphate (>90%) is in the metabolic pool. Non-metabolic phosphate is presumably stored in the vacuole, and is not readily accessible to the tissue, so that growth normally occurs at the expense of external phosphate. During deficiency, growth is limited by the rate at which phosphate can be transported through the tonoplast and tissue to the growing point. Growth ceases when the supply of non-metabolic phosphate is exhausted. Metabolic phosphate is presumably located in the cytoplasm; it can not be used for growth. Nor can the plant respond to deficiency by making some phosphorus compounds at the expense of others. In this respect, phosphorus deficiency and nitrogen deficiency are dissimilar.

Materials and Methods

Culture of Tissue and Measurement of Growth. Spirodela oligorrhiza (Kurz) Hegelm. was grown in axenic culture (3). Minus-P medium was made up: it contained 4 mM (NH₄)₂SO₄, 2 mM CaSO₄, 2 mM MgSO₄, 1 mM K₂SO₄, minor and trace elements, and 1% glucose. Control medium (3) was obtained by adding KH₂PO₄ at 1 mM to the deficient medium; and labeled medium. by adding KH₂³²PO₄ of the appropriate specific activity. Aliquots of 20 ml within 50 ml conical flasks were autoclaved and sterile CaCO₃, about 50 mg, was added to each flask to stabilize the pH at 7.4. When required, filter-sterilized kinetin (at 1 part per million) or zeatin (0.1 part per million) was added to each flask. Fronds for inoculation were washed first in sterile distilled water, then inoculated as before (3).

Growth could be measured either by counting the number of fronds present in 1 flask at successive times, or by preparing many flasks in a treatment then harvesting some at successive times. In the latter case, plants from each flask were washed in 3 changes of distilled water during 2 minutes, then blotted and weighed.

Distribution of Phosphorus in Fractions During Deficiency. Firstly, tissue was inoculated into
labeled medium (0.06 μc ³²P per μmole P per ml, at Day 0) and grown for 8 days (4 generations) to allow it to become uniformly labeled. Then, the tissue so obtained was used to inoculate further flasks containing aliquots of either the original labeled medium (control) or of a minus-P medium. Control and minus-P samples which resulted thus had all their phosphorus compounds labeled to the same specific activity, and the amount of phosphorus in any fraction could be determined from its radioactivity.

Samples were harvested after various times on the control and minus-P media; and were washed, blotted and weighed. All or a weighed part of each sample, 0.01 to 0.100 g fresh weight, was dropped into 2.5 ml methanol:chloroform:formic acid:water, 12:5:1:2 v/v (MCF) at −78° and transferred to −25° then held overnight to inactivate phosphatases (1). In order to prevent any loss of ³²P, during extraction and precipitation, 5 μmoles P, carrier was added to each sample, prior to extraction. Tissue and MCF were ground together in a Dull conical glass homogenizer (Kontes) and transferred to a 12 ml conical centrifuge tube. The homogenizer was washed out with 1 ml MCF and the washings were added to the centrifuge tube, the total homogenate was centrifuged and the supernatant was transferred to a second centrifuge tube. The residue was resuspended in 2 ml 1 % formic acid in 20 % methanol and shaken for 5 minutes then centrifuged again. Meanwhile, to the first supernatant were added 0.7 ml chloroform then 1 ml water; and the 2 phases which formed were separated by centrifuging. The colored chloroform phase, containing the phospholipids, was transferred by pasteur pipette to a planchet for counting. The second supernatant was added to the tube containing the aqueous phase and the combined extract was dried (at 40° under vacuum on a Buchler Evapomix), while the tissue residue containing the RNA was transferred to a planchet for counting. The dried extract was redissolved in 3 ml water, and P₁ was precipitated by adding 1 ml of 1 N HClO₄;10 % ammonium molybdate:0.2 M triethylammonium chloride, 4:2:1 v/v mixture (10). After centrifuging, an aliquot of the supernatant, containing the phosphate esters, was plated on a planchet; then the precipitate, containing the P₁, was dissolved in 2 N NH₄OH and an aliquot was plated. The amount of phosphorus present in each fraction was calculated from its radioactivity.

Effect of Deficiency on Phospholipid and Phosphate Ester Patterns. ³²P-labeled control and minus-P tissues were prepared in the manner already described, but at a higher specific activity, 0.5 μc per μmole P₁ per ml, and with a smaller control inoculum (3 fronds). Plants were harvested after 12 days growth on control and minus-P media, then 0.5 g tissue was killed in 10 ml MCF. It was homogenized and centrifuged, the supernatant was separated into 2 phases by addition of 2.5 ml chloroform and 3.5 ml water, and the chloroform phase containing the phospholipids was taken for study while the aqueous phase was held. The tissue residue was reextracted with 5 ml 1 % formic acid in 20 % methanol and the extract was added to the previous aqueous phase and kept. Then the residue was again extracted, with 5 ml 5 M NH₄OH, and finally discarded, the final extract being dried down at 40° under vacuum to remove ammonia. The combined aqueous extract was then added and in turn dried down to remove formic acid. The resulting dried extract contained all the simple phosphate esters and traces of RNA; it was re-dissolved in water then divalent cations were removed on a cation exchange column (5).

Phospholipids were separated by 2-dimensional thin layer chromatography (TLC) on mixed silica: cellulose (5:2 w/w layers in chloroform:methanol: water 65:25:4 v/v, then in methyl isobutyl ketone:acetic acid:water 8:5:1 v/v) (3). Phosphate esters were separated by 2-dimensional TLC on acid-washed cellulose layers in n-propanol ammonia:water:EDTA 6:3:1:0.01 v/v/v/v (twice), then in n-propyl acetate:formic acid:water 11:5:3 v/v (twice) (2).

Turnover Rates of Phosphate Esters During Phosphorus Deficiency. Unlabeled phosphorus-deficient plants were prepared by growing Spirodela on minus-P medium for 14 days. Unlabeled control plants were grown on control medium, but in order to avoid exhausting the supply of nutrients, they were subcultured onto fresh control medium at day 7. Tissues were washed, blotted, and weighed into 0.10 g samples. Each sample was put into a flat bag made from 1.5 mm nylon mesh. Samples were then pulse-labeled with ³²P, by dipping each set of 8 bags into solutions, according to the following schedule: (i) 5 minutes in 10⁻⁵ M KH₂PO₄ to fill adsorption sites. (ii) 5 minutes in distilled water to remove phosphate from the apparent free space (then blot to remove surface water). (iii) 25 seconds in 10⁻⁵ M KH₂PO₄, 0.5 μc/ml, to label the tissue. (iv) 2 seconds wash in a stream of distilled water to remove surface ³²P. (v) 3 seconds (sample 1) or 33 seconds in 500 ml 10⁻⁴ M KH₂PO₄, to remove and dilute any remaining unabsorbed ³²P. (vi) 1 minute (sample 3) to 239 minutes (sample 8) in distilled water to complete the labeling of the tissue phosphate esters.

A bag containing a 0.10 g sample of tissue, was taken at each of the following times: one-half, 1, 2, 4, 10, 30, 90, and 240 minutes after the commencement of labeling [(iii)]. The bag was slapped onto filter paper to remove excess liquid and then dropped into liquid nitrogen. The frozen fronds were later transferred to 4 ml MCF at −25° and extracted as described in the previous section. The phosphate esters were separated by 2-dimensional TLC, as before, and the radioactivity of each was measured.
Results

Changes in Growth During Onset of Phosphorus Deficiency. When phosphate-sufficient plants were transferred to a minus-P medium, their growth rate fell progressively to zero. A small but significant decrease could be detected within 8 hours of the transfer (fig 1). A second marked decrease in growth rate was detected after 2 days. Later, the physical appearance of the plants altered. Roots became much longer and newly formed fronds became progressively smaller. After 8 days, anthocyanin started to accumulate in the tissue. After 12 days, the oldest fronds became yellow, and the clumps of fronds started to break up into smaller units. After 15 days, growth stopped (fig 2). During the next 14 days the oldest fronds turned white and the next generation of fronds became yellow, but the youngest and smallest fronds remained a dark, almost black, green.

Growth of control plants also slowed down after 12 days, when the tissue weight per flask had reached 0.5 g. At this stage, both $P_1$ and $NH_4^+$ in the medium had become exhausted, (because growth of this tissue was also limited by nitrogen supply, some symptoms of phosphorus deficiency did not develop). Kinetin and zeatin each stimulated the growth of control plants, and hastened the appearance of these nitrogen deficiency symptoms. The growth rate of phosphorus-deficient plants was not affected, but symptoms of phosphorus deficiency were markedly reduced. Though anthocyanin was still formed, the roots did not elongate, the clumps of fronds did not break up, and the older fronds did not become yellow or white. Apparently, the cytokinins retarded senescence which was otherwise induced by phosphate deficiency.

Distribution of Phosphorus in Phosphorus Fractions During Deficiency. Phosphorus present in the tissue was divided into 4 fractions — $P_1$, phosphate esters, phospholipids and RNA—and the change in each during onset of phosphate deficiency was studied (fig 3). The most marked effect was on the $P_1$ content (fig 3A). It fell from 30 $\mu$moles/g fresh weight on Day 0 to 8 $\mu$moles/g on Day 4, and to 3 $\mu$moles/g on Day 9. By Day 19, when growth had ceased, the level had dropped to 0.7 $\mu$ mole/g, where it remained. The phosphate ester content was not so markedly affected (fig 3C), dropping from 3.5 $\mu$moles/g on Day 0 to 2.0 $\mu$moles/g on Day 4 and 1.3 $\mu$moles/g on Day 9; after Day 19 the level, 0.6 $\mu$ mole/g, decreased only slightly. RNA (fig 3B) and phospholipid (fig 3D) contents were even less affected, dropping from 7.5 and 3.5 $\mu$moles/g on Day 0 to 7.0 and 3.0 $\mu$moles/g on Day 4, 4.0 and 2.0 $\mu$moles/g on Day 9 and 2.0 and 1.2 $\mu$moles/g by Day 19. Thus by the time terminal phosphorus deficiency was reached the $P_1$ content had dropped to 2%, the ester phosphate content to 13%, the RNA content to 24% and the phospholipid content to 32% of the control values. Similar
Fig. 3. Effect of zeatin and phosphate deficiency on levels of phosphorus in fractions of Spirodela tissue. A) Inorganic phosphate. B) RNA fraction. C) Phosphate esters. D) Phospholipids. Curves show μmoles phosphorus/g fresh weight of tissue in the various fractions from tissue grown in: — ●—, control medium; — ●—, control medium plus 0.1 parts per million zeatin; — Δ—, minus-P medium; — △—, minus-P medium plus 0.1 parts per million zeatin. Arrows mark the time at which the P in the control media became exhausted.

Table I. Amount and Proportion of Phosphate Esters in Ester Extracts from Control and Phosphate-Deficient Spirodela

Minus-P tissues harvested after 12 days of deficiency. Results are means from two experiments. UDPX is as described in (3). U is an unknown compound chromatographing near UMP.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control tissue</th>
<th>Minus-P tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P, μmole/g tissue</td>
<td>% total P-ester</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>665</td>
<td>21.1</td>
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<tr>
<td>Mannose-6-P</td>
<td>135</td>
<td>4.9</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>120</td>
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<tr>
<td>Glucose-1-P</td>
<td>50</td>
<td>1.6</td>
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<tr>
<td>ATP</td>
<td>240</td>
<td>10.8</td>
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<tr>
<td>UTP</td>
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<td>5.1</td>
</tr>
<tr>
<td>ADP</td>
<td>140</td>
<td>4.5</td>
</tr>
<tr>
<td>UDP</td>
<td>115</td>
<td>3.7</td>
</tr>
<tr>
<td>UDPglucose + UDPX</td>
<td>210</td>
<td>6.7</td>
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<tr>
<td>UMP</td>
<td>110</td>
<td>3.5</td>
</tr>
<tr>
<td>Phosphoglyceric acid</td>
<td>325</td>
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</tr>
<tr>
<td>Phosphoenolpyruvate</td>
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<td>Phosphoryl choline</td>
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<td>Phosphoryl ethanolamine</td>
<td>60</td>
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</tr>
<tr>
<td>α-Glycerophosphate</td>
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<td>0.8</td>
</tr>
<tr>
<td>Hexose diphosphates</td>
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<td>9.7</td>
</tr>
<tr>
<td>U</td>
<td>30</td>
<td>0.9</td>
</tr>
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<td>Other compounds</td>
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<td>Total organic phosphate</td>
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<td>905</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>28,500</td>
<td>905</td>
</tr>
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</table>
changes occurred when zeatin was included in the minus-P medium. In control tissues the fractions did not alter significantly until day 9 or 10, by which time the P₁ and nitrogen supplied in the medium had become exhausted. Subsequent changes were rather similar to those encountered during the first 2 days of phosphate deficiency, except that the RNA content was more markedly affected, particularly in the presence of zeatin.

Distribution of Phosphate Esters and Phospholipids During Phosphate Deficiency. During 10 days of phosphate deficiency, the phosphate ester content of Spirodela fell to about 40% of its control value. When the phosphate ester extracts were chromatographed and the amount of each ester was estimated (table I), it was found that each had decreased in proportion, so that the pattern of the phosphate esters was substantially unaltered. Only 2 consistent differences were noted. There was a slight decrease in the hexose phosphatenucleotide phosphate ratio; and a new compound U appeared in the chromatogram immediately below P-glyceric acid and above UMP. This compound, which has not yet been identified, may be related metabolically to UMP.

In a similar way, the phospholipid content fell to about 40% during thirteen days of phosphate deficiency, and it was again found that each phospholipid decreased in proportion, so that the pattern was largely unaltered (table II). The only consistent difference was a decrease in the proportion of phosphatidyl glycerol and a slight increase in that of phosphatidyl inositol.

Turnover Rates of Phosphate Esters During Phosphorus Deficiency. When Spirodela tissues were pulse-labeled for 25 seconds with ³²P₁, the various esters became labeled at different rates (fig 4). The half-times of labeling for the various esters were estimated from the fitted curves (4). Values obtained for the various esters in minus-P tissue were a half to two-thirds the control values (table III). In both tissues, labeling times of the various phosphate esters showed a relationship to one other very similar to that found for phosphate esters in potato tissue (4). Thus ATP and UTP became labeled within the duration of the ³²P₁ pulse, 0.5 minutes, and much more rapidly than any of the other compounds. The esters of the glycolytic pathway showed labeling times similar to one another, 3 to 5 minutes in minus-P tissue and 4 to 10 minutes in control tissue. P-choline, P-ethanolamine and α-glycero-P, presumably all involved in phospholipid biosynthesis, were also fast to label, 3 to 5 minutes in minus-P tissue and 4 to 10 minutes in

![Fig. 4. Log/10 p'lot of change in percentage radioactivity with time, for various phosphate esters from control tissue (4A. left) and minus-P tissue (4B. right) which had been fed a 25-second pulse of ³²P₃-phosphate. — O —, inorganic phosphate; — ● —, glucose-6-P; — ■ —, ATP; — ● —, mannose-6-P; — △ —, UDPglucose; — ▲ —, UMP.](https://www.plantphysiol.org)
control tissue. Both UMP and U (the compound which separates chromatographically near P-glyceric acid in extracts from minus-P tissue) had labeling times of 90 minutes or longer.

**Discussion**

The visible symptoms of phosphorus deficiency in *Spirodela* (long roots, yellowed older fronds, small size of new fronds, and formation of anthocyanin leading to a blackish color in new fronds) are very similar to those noted for land plants (6,8,12,13). The marked reduction by cytokinins of the first 2 symptoms is therefore worthy of note. The earliest changes in phosphate content of *Spirodela* tissues are also parallel to those observed for other plants, in that the P1 content declines much more rapidly than does the organic phosphate content (6,7,9). In the present study, the course of deficiency has been studied in more detail, and was allowed to progress until growth of new fronds depended entirely on phosphate released by death of old fronds. At this stage, P1 was still present in the tissue; indeed, it accounted for as much phosphorus as that in all the metabolically active phosphate esters. It seems as if a part of the P1 in the
tissue can not be utilized to form esters, even under extreme deficiency stress. What is the nature of this inovative P? Though an obvious explanation is that it is irreversibly bound in some way, to cell structures or as insoluble salts, the evidence rather suggests that it is metabolically much more active than the part of P that was used to support deficient growth. This evidence will be discussed later.

Considering the intensity of the phosphate deficiency stress, relatively little change occurred in the levels of the 3 main organic phosphate fractions (phospholipids, simple esters, and RNA). A part of the slow decrease which continued in the third and fourth week of deficiency was undoubtedly due to the increasing presence of dead and dying fronds. The stability of the phosphate pattern was further emphasized when individual compounds were considered. Neither the phosphate ester pattern nor the phospholipid pattern was markedly affected by 11 to 15 days of deficiency. Other experiments not reported here have amply confirmed this observation. When pulse-labeling experiments were used, the course of labeling of the various phosphate esters was also found to be unaffected by phosphorus deficiency (table III). The results taken together show that the whole network of metabolically active phosphate esters behaves as a unit when faced by this extreme stress. In this respect, phosphate nutrition contrasts with nitrogen nutrition, where gross alterations in amino acid pattern are produced by nitrogen deficiency.

If the various phosphate esters became labeled in the same order in the 2 tissues, presumably the same metabolic paths were operating. Was the rate at which the various reactions took place altered? Turnover rates (k) for each ester can be calculated from the half-times of labeling (t) by the relationship k = (ln 2)/t (4). In minus-P tissue they were 1.5 to 2 times faster than in control tissue (from table III). As the concentration of each ester was lower in the minus-P tissue, one- to two-thirds the control value (from table I), the amount of ester being turned over per unit time per unit of tissue was of the order of three-quarters that in the control tissue. In summary, during deficiency the metabolic machine becomes smaller, but retains its form: it continues to function in the same way, but by operating at a somewhat faster rate it appears to partially compensate for its smaller size.

A more detailed analysis of data from figure 4, table I and table III shows that the P in which is in normal Spirodela must occur in at least 2 distinct pools. Consider first, control tissues that were given 32P in a very brief pulse: nearly all esters reached steady-state radioactivity values after about 30 minutes (fig 4, table III). Since the amount of each ester which was present in exactly comparable tissue is known (table I), specific activities can be obtained by dividing the steady-state radioactivity for each ester in the pulse-labeled tissue (table III) by the amount of that ester (table I). It is found that specific activities of all major esters except ADP and UDP fall in the range 120 to 205 cpm/mumole P (mean, 170), and suggesting that all esters became labeled from a common pool of P, of specific activity 170 cpm/mumole P. However, the observed specific activity of the total P in the tissue was only 22.5 cpm/mumole, and so this could not in itself have given rise to the esters. Thus the observed low specific activity of P must represent an average value derived from a small pool (or pools) of high specific activity and a large pool of low activity. If the metabolically functional pool, which has the specific activity 170 cpm/mumole, contains a fraction x of the P, and the non-metabolic pool contains the fraction 1 - x, of specific activity y, then

\[ 170x + y(1-x) = 22.5 \]

If y is zero, x has its maximum value, 0.13; if y is not, x is smaller. One line of reasoning suggests that y is very small. An interpretation of the results is that 32P entering the tissue goes exclusively into the metabolic pool, and this slowly equilibrates with the non-metabolic pool. An observed increase in the proportion of 32P in the P fraction of pulse-labeled tissue after 4 hours (fig 4A, P curve) indicates that such an equilibration does occur, and gives an idea of the time-scale. Final equilibrium would be expected to be reached when 90% of the activity was in P (from table I), but in 4 hours the percentage activity had gone from a minimum of 56% to only 64%. It must thus therefore take at least a day for the 2 pools to come into equilibrium, which in turn indicates that the specific activity of the non-metabolic pool at 1 hour could not have been greater than \( y = 3 \text{ cpm/mumole} \); and thus that the fraction of the P in the metabolic pool, \( x = 0.12 \). This result is similar to one obtained for potato tissue (4).

When data for phosphate-deficient tissue are examined, a totally different situation is encountered. Specific activities for all major esters except ADP and UDP fall in the range 525 to 890 cpm/mumole P (mean, 695 cpm/mumole), but in this case the observed specific activity of the total P in the tissue is virtually the same, 735 cpm/mumole. Therefore, if any non-metabolic pool is present, it must be very small, and virtually all the tissue P must be in the metabolic pool. The ratios, metabolic P/ester phosphate (1.08 in control, 0.82 in minus-P) will then be very similar in the 2 tissues, despite the big difference in total P/ester phosphate ratios. We can now draw a picture of what happens during phosphate-deficient growth. The non-metabolic P is progressively utilized, but the metabolic P is linked in its behavior to the phosphate esters, and decreases only slowly. When non-metabolic P has disappeared, growth ceases, for the remaining metabolic P can not be utilized for growth. Though these 2 pools are physiological entities, it is logical to attempt to assign them sites in the cell—the non-metabolic pool in the vacuole, the metabolic pool...
in the cytoplasm. P, located in the cytoplasm would therefore be an obligatory component of the cytoplasm.

The growth data (fig 1, 2) can now be interpreted in the light of these findings. Definite inhibition of growth could be detected within 8 hours of transferring control tissue to minus-P medium, and this became marked after 2 to 3 days. Yet after 8 hours, the concentration of P, in the tissue was almost unchanged (from 28, to 25 mM). Even after 2 days, tissue P, concentration was still 15 mM, and thus 15 times that supplied in the external medium to control tissue. However, the P, which was required for growth needed to be retrieved from the non-metabolic pool in the vacuole; and the rate of growth could have been restricted by the rate at which P, could be transported across the tonoplast into the cytoplasm to support synthesis of new RNA, etc. The time required for metabolic and non-metabolic pools to come into equilibrium indicates that this tonoplast barrier operates effectively. Vacuolar phosphate in control tissues may well represent a trust account that is not touched during normal growth. New growth would occur using newly absorbed phosphate taken directly into the metabolic pathway from the external environment. This raises the possibility that transient phosphate deficits could occur in metabolically active parts of the tissue even while phosphate supply within the tissue as a whole was completely adequate. It is hoped to explore some of these problems.

Acknowledgments

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Literature Cited