Possible Interference by an Acid-stable Enzyme during the Extraction of Nucleoside Di- and Triphosphates from Higher Plant Tissues

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ABSTRACT

Acid extracts from tissues of two solanaceous plants were found to contain a heat-labile, nondialyzable factor which hydrolyzes nucleoside di- and triphosphates to nucleoside monophosphates. This acid-resistant factor shows optimal ATP-hydrolyzing activity at pH 5, whereas practically no activity was detected below pH 3 and above pH 9. It does not hydrolyze sugar phosphates, nucleoside monophosphates, uridine di-phosphoglucone, and phosphoenolpyruvate. In order to estimate quantitatively the amount of nucleoside di- and triphosphates in a plant extract, care must be taken to circumvent possible interference by this factor. This is achieved by carefully maintaining the extract below pH 3.

A method commonly employed for quantitative analysis of metabolic intermediates and products from animal tissues includes (a) quick fixation of tissue by freezing; (b) extraction and deproteinization of frozen tissue with perchloric acid; (c) adjustment of the pH of extracts to near neutrality with KOH or K2CO3; and (d) quantitative determination of intermediates in neutralized extracts with a sensitive technique (1, 21). Similar procedures have been employed for extraction from plant tissues, where the intermediates have been frequently assayed with chromatographic techniques (4, 8, 9, 11, 13). Some investigators claim that this simple, rapid sampling and assay technique is not applicable to higher plant tissues because of possible degradation of labile phosphate esters in acid extracts (3, 5, 20), possible co-precipitation of phosphate esters with potassium perchlorate (2, 3), and difficulty in resolving these esters chromatographically (3, 5). Inability of acid to destroy some enzymes completely (6, 7, 21) may lead to erroneous results, when the acid extraction procedures are used.

In our studies with tobacco pith tissue (16), where direct enzymic analyses of intermediate compounds (cf. 1, 21) were performed on extracts prepared with ethanolic formic acid (cf. 5), quantitative recoveries of ATP and ADP were not as satisfactory as those obtained from extracts prepared with formic or perchloric acid alone. Acid extracts, however, showed a time-dependent destruction of ATP after their preparation. For this reason, several parameters associated with acid extraction of higher plant tissues were examined carefully. This communication reports a simple method for avoiding the activity of the adenosine di- and triphosphate-destroying factor in acid extracts.

MATERIALS AND METHODS

Plant Materials. Pith tissue isolated from mature, nonflowering tobacco plants (Nicotiana tabacum cv. Wisconsin 38) grown in the greenhouse of the Matthaei Botanical Gardens of the University of Michigan was used as the principal material for this study. Coleoptiles of dark-grown corn (Zea mays), coleoptiles, leaf tips, and stem segments of oat (Avena sativa cv. Victory), tubers of white potatoes (Solanum tuberosum) purchased from a local market, and etiolated hypocotyls of mung bean (Phaseolus aureus cv. Jumbo) were used to verify the results obtained with tobacco pith studies.

Plant tissues for extraction were prepared in the following manner. Tissues were first frozen in liquid N2, lyophilized to complete dryness, and pulverized into fine powder with a glass mortar and pestle. Either lyophilized or pulverized tissue can be stored desiccated in the dark (over a year) without noticeable change in adenylate content. Recovery of ATP and ADP from lyophilized, pulverized tissue extracted with PCA2 was compared with the extraction of these adenylates from fresh tissue which was ground in cold PCA. Both methods yielded similar results. Because of ease in handling and storage, lyophilized and pulverized tissues were used as starting material for these experiments.

Chemicals. All chemicals used were of analytical reagent or the best commercially available grade. Nucleotides, phosphorylated compounds, thin layer materials, and enzymes were purchased from the following sources: ATP, ADP, AMP, PEP from Calbiochem; UTP, UDP, UMP, CTP, CMP, GTP, GDP, and NADH from Sigma Chemical Co.; UDP-gucose, glucose-6-P, and NADP from Boehringer Mannheim Corp.; PEI from Pfaltz and Bauer, Inc.; cellulose powder MN 300 for TLC (without binder) from Macherey, Nagel and Co.; and hexokinase (EC 2.7.1.1), pyruvate kinase (EC 2.7.1.4), adenylate kinase (EC 2.7.4.3), lactic dehydrogenase (EC 1.1.1.27), glucose-6-P dehydrogenase (EC 1.1.1.49) from Boehringer Mannheim Corp.

Enzymic Determination of Adenylates and Glucose-6-P Content. Concentrations of ATP, ADP, AMP, and glucose-6-P in the extracts were determined enzymically using an Eppendorf fluorometer connected to an Esterline-Angus recorder, model S-6015 (6, 7, 21). Glucose-6-P and ATP were assayed with glucose-6-P dehydrogenase and hexokinase; ADP and AMP were measured with lactic dehydrogenase, pyruvate kinase, and adenylate kinase. The reaction mixtures and the necessary standard were prepared as recommended by Williamson and Corkey (21).

Thin Layer Chromatography. Thin layer plates (20.2 × 20.2

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2 Abbreviations: PCA: perchloric acid; PEP: phosphoenolpyruvate; PEI: polyethylenimine.
Thirty g of cellulose powder and 200 ml of the dilute PEI from Randerath and Randerath were mixed in a Waring Blender. Approximately 23 ml of the resultant slurry was spread on each plate using a glass rod and gentle rotation to obtain an even layer. Grooves were cut in the base of the plate to accommodate a small lateral expansion of the layer during prewashing (see ref. 15). Prewashing was done by developing the plates with distilled H₂O to the upper edge and air drying. Extracts were spotted 2.5 cm above the bottom edge of the plate and developed with 0.35 M or 1 M LiCl.

**PCA and HCl Extraction Methods.** The extraction procedure, somewhat modified from the method for animal tissue (cf. 21), involves the following four steps (unless otherwise mentioned, all steps were carried out at 0-4°C): (a) after careful weighing, lyophilized, pulverized plant tissue was uniformly suspended in 0.5 to 0.8 N cold PCA or 0.1 to 1 N HCl at a tissue concentration of 10 to 20 mg/ml acid; (b) the mixture was allowed to stand for 10 min with occasional shaking and centrifuged at 10,000g for 10 min; (c) a measured volume of the supernatant was pipetted into a test tube, to which were added, in sequence, a 1/16 volume of 1 M glycine solution and a given volume of 3 N KOH or K₂CO₃ to raise the pH of the extract to 2.5 ± 0.3; (d) the mixture was centrifuged at 10,000g for 10 min to obtain the clear supernatant for enzymic assays. With this method, re-extraction of the pellet from step (b) was found unnecessary. Glycine was added in step (c) to ease the pH adjustment (see "Results"). In all steps, solution volumes were recorded to facilitate the final dilution calculation (21).

**Formic Acid Extraction Method.** With the PCA and HCl procedures, high concentrations (60 mm or higher) of salts facilitate the final dilution calculation (21). A measured amount of lyophilized tissue powder was uniformly mixed with cold 1.2 to 2.4 N formic acid to give a final tissue concentration of 10 to 20 mg/ml acid. The mixture was allowed to stand for 10 min with occasional shaking and centrifuged at 10,000g for 10 min. A measured volume of the supernatant was carefully pipetted into a test tube, quickly frozen in liquid N₂, and lyophilized to complete dryness, thus removing the formic acid. The residue in the test tube was then solubilized in a small volume of 1 to 5 mM glycine-HCl buffer at pH 2.5. This volume may be either more or less than the original extract volume. Any turbidity in the resultant solution was removed by centrifugation at 10,000g for 10 min. The supernatant was ready to be assayed by an enzymic or a chromatographic method. Similar to the PCA and HCl procedures, re-extradition of the first pellet did not yield additional extraction of nucleotides and solution volumes were recorded at each step for the final calculation of the results.

**RESULTS**

**pH Effect.** Our procedures emphasize the maintenance of the extract pH below 3 during extraction steps. This precaution was derived from experiments with extracts from tobacco pith tissue. Figure 1 shows the effect of pH on the stability of ATP in tobacco pith extracts. The pH of the extracts was adjusted to a given value with KOH during step c of the PCA procedure, and the ATP content was determined enzymically at various subsequent times. The results (Fig. 1A) show that the ATP content decreases in extracts where the pH values are within the range between 3 and 9, while practically no loss of ATP is detected, if the pH values are below 3 or above 9, even 45 hr after preparation of the extracts. In order to show the pH effect more clearly, the ATP levels obtained between 7.5 and 9.5 hr are replotted in Figure 1B together with the amounts of AMP detectable between 10 and 12 hr after extraction. This figure points out that the ATP destruction is most rapid at pH 5, the amount of ATP lost is recovered nearly stoichiometrically in the form of AMP, and the pH of extract should be adjusted to below 3 or above 9 in order to avoid ATP breakdown. The following considerations caused us to choose the pH range of 2.5 ± 0.3 rather than the values above 9: (a) plant extracts often turn brown when their pH exceeds 6, suggesting the presence of phenolics which tend to interfere with the subsequent fluorometric enzymic assays; and (b) at pH values less than 1, the amount of extractable ATP decreases with time (see below), while the extracts maintained at pH 2.5 retained a high level of ATP for over 45 hr (Fig. 1A).

Table I illustrates the effects of pH at 2.5 and 5 on the stability of ATP in the PCA extracts from several higher plant tissues. The recovery of exogenous ATP added at a concentration of about 1 nmol/mg of tissues, dry weight was also examined. For both pH values, ATP content was determined immediately (0-5 hr) and 1 day (20-30 hr) after the preparation of extracts. The results for pH 5 extracts assayed immediately after preparation are not presented in this table. The pH 5 extracts from tobacco pith, tobacco leaves, and white potato tubers are clearly capable of destroying ATP, whereas other extracts show little ATP loss even after 20 to 30 hr. In all tissues tested, the extracts whose pH was adjusted to 2.5 show practically no loss of ATP in 1 day. The recovery of added ATP ranged from 78 to 108%. Thus, the pH adjustment to approximately 2.5, but not above 3, results in the maintenance of original ATP levels for long periods of time. Although the data are not presented, the results with formic acid extraction were essentially the same as for PCA extraction.
Table 1. Effect of pH 2.5 and 5.0 on ATP Stability and Recovery in PCA Extracts of Several Plant Tissues

<table>
<thead>
<tr>
<th>Tissue Extracted</th>
<th>ATP Added for Recovery</th>
<th>ATP Detected in Extract</th>
<th>Recovery of Added ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 2.5</td>
<td>pH 5.0</td>
</tr>
<tr>
<td></td>
<td>After 0-5 hr</td>
<td>After 20-30 hr</td>
<td>After 0-5 hr</td>
</tr>
<tr>
<td>nmol/mg dry wt</td>
<td>nmol/mg dry wt</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Tobacco pith</td>
<td>0</td>
<td>0.69</td>
<td>0.66</td>
</tr>
<tr>
<td>Tobacco leaves</td>
<td>1.15</td>
<td>1.84</td>
<td>1.81</td>
</tr>
<tr>
<td>White potato tubers</td>
<td>0.83</td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td>Mung bean hypocotyl</td>
<td>0.96</td>
<td>0.94</td>
<td>1.02</td>
</tr>
<tr>
<td>Corn coleoptile</td>
<td>1.00</td>
<td>1.16</td>
<td>1.17</td>
</tr>
<tr>
<td>Oat coleoptile</td>
<td>1.56</td>
<td>1.60</td>
<td>1.42</td>
</tr>
<tr>
<td>Oat first leaf tips</td>
<td>1.10</td>
<td>2.67</td>
<td>2.64</td>
</tr>
<tr>
<td>Oat p-1 stem</td>
<td>0.08</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>segments</td>
<td>0.64</td>
<td>0.59</td>
<td>0.58</td>
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<tr>
<td></td>
<td>0.57</td>
<td>0.56</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>1.39</td>
<td>1.72</td>
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<tr>
<td></td>
<td>0.67</td>
<td>0.71</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Concentration of Extractant. The effect of PCA concentrations on ATP and glucose-6-P extraction was examined in tobacco pith extracts adjusted to pH 2.5 ± 0.3. In these studies, both ATP and glucose-6-P determinations were made at two different times after the preparation of extract. The results (Fig. 2) clearly show that PCA concentrations higher than 0.1 n completely extract ATP and glucose-6-P, and the amounts remained constant for more than 30 hr after extraction. Although the results are not shown, HCl above 0.1 n or formic acid above 1.2 n was also found to extract ATP and glucose-6-P fully. In all of these experiments, the extract made in the absence of acid contained very little ATP, whereas the concentration of glucose-6-P in water extracts was about 90% of the level in acid extracts (Fig. 2).

In our procedure with a relatively short period of extraction (10-30 min), the initial acid concentration should be such that the pH of the extract is kept below 1 for PCA and HCl and 1.8 for formic acid to insure complete extraction. The use of high concentrations of PCA and HCl resulted in solubilization of starch (16), unnecessary dilution of the extract during pH adjustment, and an increase in background and quenching of fluorescence in fluorometric assays. For these reasons, the following concentrations of acids are used in our extraction routinely: 0.3 to 0.8 n PCA, 0.2 n HCL, and 1.2 to 2.5 n formic acid.

Other Parameters. The PCA and HCl extraction time (i.e. the time between the introduction of the acid to the powdered tissue and the adjustment of pH to 2.5 with alkali) is about 30 min. Similarly, the extraction time with the formic acid procedure (the time between acid addition and freezing of extract in liquid N₂) is 10 min. In one series of experiments with tobacco pith tissues, an extreme extraction time of 25 hr gave values of ATP about 40% less and of glucose-6-P about 25% less than the standard procedure. In another, 8 nmol PCA completely inactivated a commercial preparation of wheat germ phosphatase in 20 min. Extracts of tobacco pith made with PCA and formic acid contained no detectable activities of adenylyl kinase and malate dehydrogenase. From these observations, the extraction times as stated in "Materials and Methods" were derived.

Our procedures do not include a re-extraction step. If the ratio of the lyophilized tissue powders to the extractant acid volume is maintained at 10 to 20 mg/ml or less, re-extraction of the first pellet yields no more ATP and glucose-6-P than can be accounted for by the volume of extract carried over in the pellet. Although the data are not presented, this observation was repeated several times with tobacco pith tissue and oat stem segments.

Nature of ATP Hydrolysis by Tobacco and Potato Extracts. Among the plants examined, tobacco and potato extracts contain the ATP-hydrolyzing factor, whereas extracts of other tissues do not (Table I). Dialysis of tobacco or potato extracts overnight at 0 C against distilled H₂O or buffer did not decrease the activity of ATP-hydrolyzing factor. On the other hand, boiling destroyed the activity of the factor, but at the same time, destroyed the ATP originally present in the extract. The destruction of ATP was not caused by a heat-stable factor, because ATP added after the first boiling was not destroyed by a second boiling. Furthermore, the extracts did not act on AMP, UDP-Glc, glucose-6-P, fructose-6-P, fructose-1, 6-dip., and PEP, but hydrolyzed ADP in much the same manner as ATP. These results, coupled with a distinct pH maximum for ATP hydrolysis (Fig. 1B), strongly suggest that the ATP hydrolysis is enzymic.

In order to examine whether the conversion of ATP to AMP (Fig. 1) proceeded with ADP as an intermediate, a high concentration of ATP (360 nmol) or ADP (340 nmol) was added to 3 ml PCA extracts whose pH was adjusted to 5; the levels of ATP, ADP, and AMP were examined at various times after the start of incubation at 0 C. The results (Fig. 3) clearly show that ATP is stoichiometrically converted to ADP + AMP. An initial ATP hydrolysis rate of 23 nmol/hr results in 10 nmol AMP and 13.5 nmol AMP formed/hr (Fig. 3A). The ADP to AMP conversion resulted in 34.5 nmol ADP lost and 33 nmol AMP formed/hr (Fig. 3B). The rate of the ADP to AMP conversion thus proceeds at 1.5 times the rate of the ATP to ADP + AMP conversion. This observation can account for the fact that the rate of

Fig. 2. Effect of PCA concentrations on extractable glucose-6-P and ATP at 4 C. Extracts for each PCA concentration tested were prepared from lyophilized tobacco pith tissue and assayed for glucose-6-P and ATP at 1.0 to 4.0 and 29.5 to 32.5 hr after preparation of extracts. DW: dry weight.
AMP formation from ATP is 35% faster than the rate of ADP formation (Fig. 3A), and also an earlier observation that ATP was stoichiometrically converted to AMP when determinations were delayed by 2.5 hr after ATP assays (Fig. 1B). These results are consistent with the idea that the conversion of ATP to AMP is carried out via ADP.

In order to examine whether the extract is capable of converting other nucleoside triphosphates to respective di- and monophosphates, formic acid extracts of tobacco pith adjusted to pH 5 were incubated with 1 mm ATP, GTP, CTP, or UTP for 30 hr at 0 C. An aliquot of each reaction mixture was used for TLC with respective controls. The results showed that most of each triphosphate incubated was converted to the corresponding monophosphate and a small amount to diphosphate (data not shown).

**DISCUSSION**

An ideal procedure for the extraction of metabolic intermediates and products from tissues should include the following considerations. (a) All enzymes should be denatured instantly with the addition of extractant, but without concomitant chemical degradation of the compounds of interest. (b) All compounds of interest should be extracted without loss in a soluble fraction. (c) The extract should be brought to a condition suitable for subsequent assay. (d) The whole procedure should involve as few and as simple steps as possible, thus maximizing the yields and recovery of the compounds (cf. 5, 21). Our results indicate that an acid extractant cannot completely denature a phosphatase-like factor which specifically hydrolyzes nucleoside di- and triphosphates, particularly when solanaceous plant tissues are used for extraction. This problem can be circumvented by maintaining the pH of the tissue extracts below 3 throughout the extraction procedure and by raising the pH to near neutrality at the time of quantitative assay. With this precaution, the acid extract was found to maintain the initial concentrations not only of nucleoside di- and triphosphates, but also of other metabolic intermediates for at least 2 days (Figs. 1 and 2; Table 1).

A plant enzyme which shows characteristics similar to the phosphatase-like factor is potato apyrase (ATP diphosphohydrolase EC 2.6.1.5). At least two isoenzymes of apyrase have been characterized on the basis of different degrees of ATPase and ADPase specificity (10, 18) and resolved by ion-exchange columns (12, 18). Furthermore, apyrase was extracted with an acid precipitation technique (17). These characteristics of apyrase are similar to those of our factor, but our data were inconclusive as to whether our factor is identical with potato apyrase or composed of more than one isoenzyme. In the literature, the presence of phosphatases has been noted in alcoholic extracts (2, 3, 5). These phosphatases carry out both hydrolysis and phosphorylation of many alcohols and other compounds (2, 13). However, these enzymes have been shown to be denatured by formic acid (2, 5). Our factor is probably different from the phosphates in the alcoholic extracts of plant tissues.

*LITERATURE CITED*