Modification of Metabolic Pattern by Variation of Nicotinamide Adenine Dinucleotide Phosphate Level

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Abstract. The experiments were designed to get some information on the metabolism controlled by variation of the NADP level, which is known to change with the variation of environmental factors.

The exogenous NADP added to the mitochondria prepared from Vigna sesquipedalis cotyledons was associated with and/or penetrated into the mitochondria. The combined NADP served in the operation of the mitochondrial NADP-isocitric acid dehydrogenase.

The variation of NADP level by exogenous NADP was observed to modify the rates of metabolic processes. The increase of exogenous NADP in Vigna hypocotyl slices lowered malic- and citric-acid contents and raised the α-ketoglutaric acid content. The incorporation of 14C from acetate-2,14C into lipid, organic acid, amino acid, was promoted with the exogenous NADP. The 14C-incorporation into glycolic acid, malic acid and glutamic acid was accelerated.

In the mannitol homogenate of Vigna cotyledon, 14CO2 evolution and 14C-incorporation into lipid, sugar, and glycolic acid from acetate-2,14C were promoted with the exogenous NADP. Endogenous citric acid content was lowered by NADP, while malic acid content was increased.

The activation of NADP-enzymes by NADP was discussed to be involved in these variations.

Nicotinamide coenzymes were previously observed to occur in various plant tissues at such a low concentration that they could be rate limiting for specific enzyme reaction sequences (18). The pattern of nicotinamide coenzymes rapidly changes with the variation of environmental factors. The variation of coenzyme pattern in relation to oxygen concentration (19), white light (8, 9), red light (Tezuka and Yamamoto, unpublished), low temperature treatment (4), auxin (7), kinetin (17), nutritional elements (10), and aging (18) have been investigated. In many cases, the variation in NADP+NADPH level was more prominent than the variation in NAD+ NADH level (4, 7, 8, 9, 18, 19). Since NADP enzymes participate in many branching points of metabolic pathways in higher plants, the variation of NADP level modifies the ratio in the flow rate between metabolic pathways (19).

A high NADP+NADPH level was observed under aerobic conditions and a low level under anaerobic conditions (19). Both white light (8, 9) and red light (Tezuka and Yamamoto, unpublished) stimulate NADP formation from NAD. Indole-acetic acid raises the NADPH/NADP ratio (7). Kinetin raised the NADP+NADPH level (17). The NADP+NADPH level and the NADPH/NADP ratio decreased accompanying organ senescence (18). The enzymological mechanism of the variation of NADP+NADPH level was previously discussed (19, 20). The NADP+NADPH variation mainly occurs in the soluble fraction of the cell components (8, 20). Previously, it was shown that addition of NADP to bean hypocotyl slices lowered the C6/C5 ratio for CO2 output in glucose catabolism (19). This decrease suggests an increased contribution to the respiratory metabolism of the pentose phosphate cycle which is known to be located in the soluble fraction. Accordingly, NADP variation can easily be supposed to modify the metabolic activities in the soluble fraction. However, there has been no evidence to prove that extramitochondrial NADP affects the mitochondrial enzymes.

The purpose of the present work was to study the effect of the NADP+NADPH level on various phases of metabolism.

Materials and Methods

Plant Materials. Seeds of Vigna sesquipedalis (L.) Fruwirth, were immersed overnight in tap water and germinated in moist sand at 30° in the dark (18).

Experiments With Vigna Hypocotyl Slices. The hypocotyls were removed from 3-day Vigna seedlings. They were cut into sections of about 0.5 mm thickness with a razor, and were washed twice with distilled water. The sections of hypocotyl were blotted lightly and thoroughly randomized. Three g (fresh wt) of the slices, 200 μmoles of potassium phosphate pH 5.0, and the indicated amount of NADP (table II, Fig. 2) in a total volume of 5.5 ml were placed in each of 3 Warburg flasks. The first received no NADP (control), while 0.5 μmole of

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1 This work was supported in part by a grant from the Ministry of Education in Japan.
NADP was added to the second flask and 1.5 μmoles of NADP to the third flask. Each flask was evacuated and air was introduced. The procedure was repeated 5 times (vacuum infiltration). Each flask was kept for 200 min in a refrigerator (3°C) to ensure that the NADP penetrated the tissues. Then, to each flask 0.5 ml (25 μmoles, 400,000 cpm) of acetate-2-14C was added. The acetate was added immediately before attaching the flasks to the manometers. The center well of each flask contained 0.5 ml of 20% KOH to absorb the respired 14CO₂. The flasks were shaken at 30°C for 60 min. The atmosphere in the flask was air.

At the end of the experiments the KOH-K₂CO₃ in the center well was removed and converted to BaCO₃, which was then washed and dried and assayed for radioactivity.

Immediately after removal of the KOH, the tissue was killed by the addition of 20 ml of boiling 80% (v/v) ethanol. The tissue was then successively extracted for 10-min periods on the steam bath with 20 ml of 80% ethanol, 20 ml of 20% ethanol, 20 ml of water, and finally, 20 ml of 80% ethanol. The extracts were filtered and combined: the insoluble residue was retained by the filter. The combined extracts were taken to dryness in a vacuum on a water bath at 40°C. The dry residue was successively extracted with ethyl ether and water, to yield, respectively, the lipid fraction and the water-soluble fraction (2).

Experiments With Vigna Cotyledon Homogenate. The cotyledons (9.0 g in fresh wt) removed from 2-day Vigna seedlings were ground with 18 ml of 0.5 mannitol, 0.05 M potassium phosphate pH 5.2 and 0.01 M MgCl₂, in a blender for 1 min at 45 v. Without filtering, the homogenate was centrifuged lightly at 1000g for 10 min. The supernatant including mitochondria was used for the following experiments.

Each of 3 Warburg flasks contained 3 ml of the supernatant (pH 5.2), 40 μmoles of nicotinamide, the indicated amount of NADP (Table III, Fig. 3), 25 μmoles (400,000 cpm) of acetate-2-14C in a total volume of 3.85 ml. The first flask received no NADP (control), while 0.25 μ mole of NADP was added to the second flask and 0.75 μmole of NADP to the third flask. The acetate was added immediately before attaching the flasks to the manometers. The center well of each flask contained 0.5 ml of 20% KOH to absorb 14CO₂. The flasks were shaken at 30°C for 3 hr. The atmosphere in the flask was air.

At the end of the experiments the KOH-K₂CO₃ was removed and converted to BaCO₃, which was then washed and dried and assayed for radioactivity. Immediately after removal of the KOH, boiling 80% ethanol was added to the flask to stop the reaction. The mixture was then transferred to a 50 ml-volume test tube. The flask was washed several times with boiling 80% ethanol. The washed alcohol solutions were also transferred to the test tube and combined with the former mixture. The combined alcohol solution (about 20 ml) was boiled for 10 min. After cooling at -5°C, the alcohol solution was centrifuged at 10,000g for 10 min. The supernatant was evaporated in a vacuum on a water bath at 40°C. The dry residue was successively extracted with ethyl ether and water, to yield, respectively, the lipid fraction and the water-soluble fraction.

Analytical Methods. The water-soluble fraction was separated into 3 fractions through the use of ion exchange resins. Dowex 50W × 8 (H) and Dowex 1 × 10 (formate). These fractions were acidic (mainly organic acids), basic (mainly amino acids), and neutral (mainly sugars). The separation was carried out in the conditions described by Canvin and Beevers (2).

The organic acid fraction was separated into its constituents by linear gradient elution on ion exchange resins (11). The volume of fluid in the mixing flask to make a linear gradient was kept at 100 ml and the reservoir contained 4 ml formic acid. The organic acids were eluted from an 11-×1-cm column of Dowex 1 × 10 (formate). The eluate was collected in 3 ml fractions and the radioactive fractions were determined by counting a 0.3 ml aliquot on a metal planchet. The remaining 2.7 ml aliquot was dried under an air stream on a water bath at 40°C. To each tube, 2 ml of CO₂-free water was added. The solutions were then titrated to the phenol red end point with CO₂-free 0.005 × NaOH delivered from a microburette (11). Acid content was expressed as μequivalents of the acid contained per ml of each 3 ml fraction. The radioactive “peaks” and the acid peaks were identified by their position of elution from the resin, by cochromatography on resin with 2 mg of authentic samples under a linear gradient of formic acid as previously described, and by paper chromatography in n-butanol-acetic acid-water (12:3:5 by volume). ethanol-ammonia-water (16:1:3 by volume).

The amino acid fraction was dissolved in 6 ml of 0.5 M acetic acid and the radioactivity of this fraction was determined by filtering a 0.2 ml aliquot on a metal planchet. The remaining 5.8 ml was placed on an 11-×1-cm column of Dowex 1 × 8 equilibrated with 0.5 M acetic acid, and the column was eluted with 0.5 M acetic acid. The eluate was collected in 3 ml fractions and the radioactive “peaks” were determined by counting a 0.3 ml aliquot on a metal planchet: amino acid “peaks” were determined with ninhydrin (22). The amino acids were identified by their position of elution from the resin and by cochromatography on paper with authentic samples in phenol-water (80:20 by volume), n-butanol-acetic acid-water (12:3:5 by volume).

The lipid fraction was combusted in a Stutz and Burris apparatus (14) using the wet combustion reagents of Van Slyke and Folch (15). Sugar fraction was converted to CO₂ by the persulfate method of Katz et al. (3). Protein content was measured by the method of Lowry et al. (5).
Results and Discussion

Association of Exogenous NADP With Isolated Mitochondria. The cotyledons (20 g in fresh wt) separated from 2-day Vigna seedlings were ground with 60 ml of a grinding medium (0.5 M sucrose, 0.1 M potassium phosphate pH 7.5, and 0.01 M KCl) in a blender for 45 sec at 45 v. The homogenate was strained through 4 layers of gauze and centrifuged at 6000g for 10 min. The supernatant was centrifuged at 10,000g for 30 min. The sediment (mitochondria) was suspended in 12 ml of the grinding medium. The particles in a 1 ml aliquot of the suspension were fixed, mounted, sectioned, and then observed with an electron microscope. The mitochondria showed typical inner membrane structures and the suspension was estimated to be at least 70% pure for the mitochondria.

The remaining mitochondria suspension was divided into two 5 ml portions. To the first 5 ml of the mitochondria suspension, 2.0 mg (about 2.5 μmoles) of NADP (Sigma) was added. The suspension was then kept for 1 hr at 0°. The mitochondria were collected with centrifugation at 10,000g for 30 min and suspended again in 10 ml of the grinding medium. This procedure for the mitochondrial washing was repeated twice more. Finally, the washed mitochondria were suspended in 6 ml of the grinding medium (4.0 mg protein per ml).

The other 5 ml of the mitochondrial suspension was similarly treated as described above in the absence of added NADP (control). The final suspension contained 4.0 mg protein per ml. The amounts of NAD, NADH, NADP, and NADPH of mitochondria in both series were measured as described previously (18). Two ml of the final mitochondria suspension from each was treated with HCl (final concn, 0.1 M) to extract the oxidized form of nicotinamide coenzymes. Another 2 ml of the suspension from each was treated with NaOH (final concn, 0.1 M) to extract the reduced form of the coenzymes.

Table I shows total amount of each coenzyme in the final washed mitochondria which were suspended in 6 ml of the grinding medium.

The contents of NAD and NADH in the mitochondria treated with NADP were almost the same with those in the control experiment, whereas the NADP+NADPH content in the NADP-treated mitochondria was 3 times higher than in the control. About 50% of the NADP newly increased in the NADP-treated mitochondria was reduced (table I). This suggests that NADP penetrated the mitochondria through their membranes and/or that NADP was associated with the mitochondrial membrane.

The NADP-NADP isocitric dehydrogenase activity by the endogenous NADP associated with mitochondria. The assay system contained the following constituents in the stated amounts in μmoles: MgCl₂, 15, isocitrate 2, tris-HCl pH 7.4, 2, 8-dichloro-phenolindophenol 0.24, mitochondrial suspension 0.1 ml (0.4 mg protein) in a final volume of 3.0 ml. Reaction temperature 22°. Fig. 1B. (right). NADP-NADP isocitric dehydrogenase activity by the endogenous NADP in the mitochondria (cf. 16).

Birt and Bartley (1) have confirmed by direct analysis that mitochondria from rat liver can exclude

Table II. Effect of NADP on Metabolism of Acetate-2-14C in Vigna Hypocotyl Slices

<table>
<thead>
<tr>
<th>NADP added</th>
<th>0</th>
<th>0.5 μmole</th>
<th>1.5 μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>14CO₂ evolution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>903</td>
<td>291</td>
<td>910</td>
</tr>
<tr>
<td>Lipid(a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>90</td>
<td>98</td>
<td>124</td>
</tr>
<tr>
<td>(specific radioactivity, cpm/mg BaCO₃)</td>
<td>3.9</td>
<td>3.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Organic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>2577</td>
<td>3591</td>
<td>3569</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmoles)</td>
<td>4.44</td>
<td>4.78</td>
<td>5.60</td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>300</td>
<td>460</td>
<td>473</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmoles)</td>
<td>2.47</td>
<td>1.71</td>
<td>1.46</td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>391</td>
<td>98</td>
<td>147</td>
</tr>
<tr>
<td>Residual amino acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmoles)</td>
<td>4.44</td>
<td>5.77</td>
<td>7.26</td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>711</td>
<td>737</td>
<td>761</td>
</tr>
<tr>
<td>Sugars(b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>213</td>
<td>414</td>
<td>401</td>
</tr>
</tbody>
</table>

(a) Wet combustion (14).
(b) Persulfate oxidation (3).

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Vigna readily anid mitochondria in lism reductionls. aind transferred ol) served can, fraction Vigna 14C-incorporation (Aequivalents/ml). NADP acetate-2-14C absence of Acids Organlic acids, NADP in the and NADI', at i-i.o FIG. hypocotyl Effect of Purvis the mitochondria rates and Purvis, in which of exogenous acids, NADP in the and NADPH changes in intra-. Metabolism in Part of NADPH and rates in mitochond(Irial membrane oxidationl participation in and NADPH be. lead to the 35 % replacement of mitochondrial NADP in about 1 hr.

The variation of NADP level in the soluble fraction can, thus, be expected to modify the metabolism in the mitochondria as well as in the soluble fraction.

Effect of NADP on Metabolism of Endogenous Organic Acids and Exogenous Acetate-2-14C in Vigna Hypocotyl Slices. Slices from 3-day Vigna hypocotyls were subjected to the incorporation of acetate-2-14C for 1 hr in the presence or in the absence of exogenous NADP. In relation to change in NADP level, the changes in quantity of lipids, organic acids, amino acids, sugars and CO2 evolved: and the changes in incorporation of 14C from acetate-2-14C into the above components were examined (table II, Fig. 2).

The increase of NADP level raised the incorporation of 14C from acetate-2-14C into lipids, organic acids, amino acids, and sugars (table II). NADP is known to participate in the biosynthesis of lipids and sugars from acetate.

The incorporation of 14C into glycolic acid was greatly accelerated under the enriched NADP conditions (Fig. 2). NADPH-linked glyoxylate reductase has been reported in spinach leaves (23). If this enzyme were present in the hypocotyl, it could contribute toward increased 14C-incorporation into glycolic acid from acetate-2-14C when NADP was added.

The 14C-incorporation into malic acid was slightly promoted. The 14C-incorporation into succinic acid and “citric” peak (citric and isocitric acids) was not altered (Fig. 2). The quantities of malic acid, “citric” peak (Fig. 2), and aspartic acid (table II) were, however, decreased with enriched NADP. On the other hand, contents of α-ketoglutaric acid (Fig. 2) and glutamic acid (table II) were increased. This may indicate that the labeled malic and citric acids newly formed from acetate-2-14C may be in a compartment different from that of endogenous non-labeled malic and citric acids which are originally present in the tissue (6). α-Ketoglutaric acid may, then, be formed primarily from the endogenous non-labeled acids in the latter compartment by enriched NADP.

The increase of NADP-isocitric dehydrogenase activity in the latter compartment by NADP will activate the conversion of non-labeled malic- and citric-acids to α-ketoglutaric acid. The increase of NADP-isocitric dehydrogenase in the former com-

Table III. Effect of NADP on Metabolism of Acetate-2,14C in Vigna Cotyledon Homogenate

<table>
<thead>
<tr>
<th>NADP added</th>
<th>0</th>
<th>0.25 μmole</th>
<th>0.75 μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>14CO2 evolution (total counts, cpm)</td>
<td>2600</td>
<td>3220</td>
<td>3106</td>
</tr>
<tr>
<td>Lipid1 (total counts, cpm)</td>
<td>532</td>
<td>548</td>
<td>726</td>
</tr>
<tr>
<td>(specific radioactivity, cpm/mg BaCO3)</td>
<td>24.4</td>
<td>25.1</td>
<td>33.3</td>
</tr>
<tr>
<td>Organic acid (total counts, cpm)</td>
<td>47,300</td>
<td>46,250</td>
<td>46,300</td>
</tr>
<tr>
<td>Glutamic acid (μmoles)</td>
<td>7.33</td>
<td>7.64</td>
<td>12.84</td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>779</td>
<td>807</td>
<td>778</td>
</tr>
<tr>
<td>Aspartic acid (μmoles)</td>
<td>1.19</td>
<td>0.80</td>
<td>0.79</td>
</tr>
<tr>
<td>(total counts, cpm)</td>
<td>361</td>
<td>331</td>
<td>223</td>
</tr>
<tr>
<td>Residual amino acids (total counts, cpm)</td>
<td>3958</td>
<td>3639</td>
<td>4130</td>
</tr>
<tr>
<td>Sugars2 (total counts, cpm)</td>
<td>2900</td>
<td>3440</td>
<td>15690</td>
</tr>
</tbody>
</table>

1 Wet combustion (14).
2 Persulfate oxidation (3).
partment by NADP will participate in the increase of 14C-incorporation into malic acid.

A number of enzymes which catalyze individual reactions of the tricarboxylic acid cycle, such as malate dehydrogenase, condensing enzyme, aconitase, and isocitric dehydrogenase are found in the soluble fraction as well as in the mitochondria (cf. 13). The soluble enzymes were shown to be different proteins from their counterparts in the mitochondria (13, 21). Though the results of the present study do not provide any clear-cut evidence, the former and the latter compartments mentioned above may be, respectively, mitochondria and the soluble fraction.

**Effect of NADP on Metabolism of Endogenous Organic Acids and Exogenous Acetate-2-14C in Vigna Cotyledon Homogenate.** Two-day Vigna cotyledons homogenized with mannitol-phosphate (pH 5.2)-MgCl2 were subjected to the incorporation of acetate-2-14C for 3 hr in the presence or in the absence of exogenous NADP (table III, Fig. 3). Malic acid content (Fig. 3) and glutamic acid content (table III) were increased by exogenous NADP, whereas the "citric" peak (citric and isocitric acids, Fig. 3) and aspartic acid content (table III) were decreased. The increase of NADP-isocitric dehydrogenase activity by NADP may be involved in these variations. 14CO2 evolution, 14C-incorporation into lipids, sugars (table III) and glycolic acid (Fig. 3) were increased under the enriched NADP level. The same mechanisms as in the hypocotyl-slices will participate in these changes.

The variation of NADP level can be concluded to play a regulatory role in a wide range of metabolism.

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**Literature Cited**