Plant Physiol. (1965) 41, 1179–1184

Increase of Mitochondrial Fraction in Sweet Potato Root Tissue after Wounding or Infection with Ceratocystis fimbriata[1]

Tadashi Asahi, Yuji Honda, and Ikuzo Uritani
Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Nagoya, Japan

Received May 16, 1966.

Summary. The acid-insoluble nitrogen content, lipid content, and cytochrome oxidase activity in the mitochondrial fraction are found to increase during incubation of slices of sweet potato (*Ipomoea batatas*) root tissue. These increases appear to be related to an increase in the number of the mitochondrial particles. The increase in the mitochondrial fraction is not accompanied by an increase in cell number. The nitrogen content in the mitochondrial fraction increases prior to the changes in the activity of cytochrome oxidase and lipid content. The increase in the numbers of the mitochondrial particles lags behind the increase in the cytochrome oxidase activity. Such findings are also found in the tissue infected by *Ceratocystis fimbriata*.

The respiratory increase in response to wounding and infection appears to be a result of an increase in mitochondrial particles.

The increase in the respiratory activity in tissue slices of tuber and tuberous root tissues has been well known as wound respiration. Hackett and his coworkers (5, 8) demonstrated that the respiration of potato tuber tissue became relatively insensitive to carbon monoxide and cyanide during incubation of the sliced tissue and that there appeared to be some differences in the content of respiratory enzymes. This indicates the complexity of the mechanism of wound respiration. Recently, it was shown by Click and Hackett (3) that the wound respiration was dependent on the synthesis of RNA and protein.

On the other hand, the respiratory increase in plant cells accompanied by the infection with various pathogens has been reported by many investigators. Reports from our laboratory (1, 9, 10, 11) have suggested that the increase of respiratory activity in storage tissues after infection with *Ceratocystis fimbriata* is due to increased availability of ADP in the tissue provided by activation of some metabolic systems.

This paper deals with the increase in mitochondrial particles in wounded and diseased tissues of sweet potato root. The results of our studies suggest that the respiratory increase in these tissues results from the increase of mitochondrial particles in the cells.

Materials and Methods

Plant Material. Sweet potato roots (variety Norin No. 1) harvested at Kariya Farm, Aichi in October were stored at 10° until used. Unless otherwise indicated, the roots were sliced in a 3.0 mm thickness after removing the surface of roots and incubated at 28 to 30° in a moisture chamber. After incubation, the whole tissue was used for the experiments as the wounded tissue.

Preparation of Mitochondrial Fraction. The minced tissue was homogenized with twice the tissue weight of 0.05 M Tris buffer (pH 7.0) containing 0.5 M sucrose, 0.01 M EDTA and 1.0 % sodium isoascorbate for 1.0 minute in a blendor. The homogenate was then squeezed through a double layer of cheese cloth and centrifuged at 300 × *g* for 10 minutes. The supernatant fraction was again centrifuged at 14,000 × *g* for 15 minutes and the resultant precipitate was suspended in 0.05 M Tris buffer (pH 7.0) containing 0.5 M sucrose. The suspension was centrifuged at 14,000 × *g* for 20 minutes and the precipitate of mitochondria was suspended in 0.01 M phosphate buffer (pH 7.0). All procedures were carried out at approximately 3°.

Determination of Components in Mitochondrial Fraction. Nitrogen content in the mitochondrial fraction was determined with Nessler's reagent by the procedure described by Johnson (7) after precipitating with trichloroacetic acid at a final concentration of 7 %. The lipid fraction extracted by the method of Folch et al. (4) was assayed for nitrogen and phosphorus.

Succinate dehydrogenase activity was assayed by the ferricyanide method (2). Cytochrome oxidase activity was determined by measuring the O_2 consumption with an oxygen electrode apparatus described by Hagihara (6). The reaction mixture was composed of 100 μmoles of phosphate buffer (pH

[1] This paper contributes part 52 of the phytopathological chemistry of sweet potato with black rot.

7.0), 30 μmoles of sodium isoascorbate, 0.06 μmole of cytochrome c and mitochondrial suspension in a final volume of 3.9 ml. The difference between the rates of O_2 consumption with and without cytochrome c was proportional to the amount of mitochondrial suspension provided that the amount of O_2 uptake did not exceed 60 mμmoles per minute.

Counting of Mitochondrial Particles. For these experiments, the final preparation described above was suspended in 0.05 M Tris buffer (pH 7.0) containing 0.5 M sucrose instead of phosphate buffer. The fraction prepared from 10 g of the tissue was suspended in 100 ml of the medium. When more concentrated suspensions were made, the particles aggregated after mixing with the dye solution. The suspension was then mixed with a half volume of 0.1 % Janus green B solution in the cold. When lesser amounts of the dye solution were used, the stained particles were hard to differentiate from the non-mitochondrial particles because staining was weak.

Immediately after mixing the mitochondrial suspension with the dye solution, 3 μl of the mixture were placed on a glass slide and the number of stained particles counted under 600-fold magnification.

Results

Increase of Acid-insoluble Nitrogen Content and Cytochrome Oxidase Activity in Mitochondrial Fractions from Wounded and Diseased Tissues. Table I shows acid-insoluble nitrogen content and cytochrome oxidase activity in the mitochondrial fractions from fresh, wounded and diseased tissues of sweet potato root. Both the nitrogen content and the enzymic activity in the fractions from the wounded and diseased tissues were found to be 4 to 7 times greater than those from the fresh tissue. The increase in the nitrogen content and enzymic activity was observed in the inner tissue to a distance of 3 to 4 mm below the cut or infected surface. It seemed likely that the tissue adjacent to the infected parts of the slices inoculated with the fungus would contain larger amounts of the mitochondrial fraction than the corresponding parts of wounded, non-inoculated slices. However, neither significant difference in the nitrogen content nor enzymic activity between the mitochondrial fractions prepared from the wounded and wounded-inoculated tissues was observed. Therefore, the wounded tissue was used in all following experiments.

Recovery of Mitochondrial Fraction from Tissues. Increasing the volume of the homogenizing

Table I. *Acid-insoluble Nitrogen Content and Cytochrome Oxidase Activity in Mitochondrial Fraction from Fresh Wounded and Diseased Tissues of Sweet Potato Root*

Sweet potato roots were sliced approximately in a 30 mm thickness and incubated for 2 days at 28° in moisture with and without *Ceratocystis fimbriata* inoculation. Then disks 1.0 mm thick and 11 mm diameter were cut from the incubated slices and used for mitochondrial preparations. The values were expressed as the amount per 10 disks, of which the total fresh weight was approximately 1.0 g.

Distance from cut surface (mm)	Wounded tissue		Diseased tissue	
	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)
0.0 — 1.0	213	113	279	112
1.0 — 2.0	194	107	224	146
2.0 — 3.0	183	114	205	129
3.0 — 4.0	196	106	184	116
fresh tissue	29	24	29	24

Table II. *Extraction of Mitochondrial Fraction with Various Volumes of Medium from Sweet Potato Root Tissue*

The values are expressed as amounts per g of fresh weight of tissue.

Volume of medium per g of fr wt of tissue (ml)	Fresh tissue		Wounded tissue	
	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)
2	21.8	18.6	56.8	70.6
5	21.2	16.0	64.8	89.1
10	21.4	10.6	68.7	55.7

Table III. *Repeated Extraction of Mitochondrial Fraction from Sweet Potato Root Tissue*

The first fraction was prepared as described in Materials and Methods. The precipitate obtained by centrifugation of the first fraction at 300 × *g* for 10 minutes was collected and homogenized again in 2 ml of medium per g of the starting sample. The mitochondrial fraction was collected from the second homogenate by the same procedures described for the first fraction and labeled the second fraction. The third and fourth fractions were obtained by repeating the same procedures. The values were as described for table II.

Mitochondrial fractions	Fresh tissue		Wounded tissue	
	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)	Acid-insoluble nitrogen (μg-N)	Cytochrome oxidase activity (mμmoles O_2 /min)
1st fraction	23.2	18.7	60.4	49.3
2nd fraction	4.3	3.9	18.1	10.2
3rd fraction	2.6	0.6	6.2	0.9
4th fraction	2.3	0.8	4.0	1.2
Total recovered	32.4	24.0	88.7	61.2

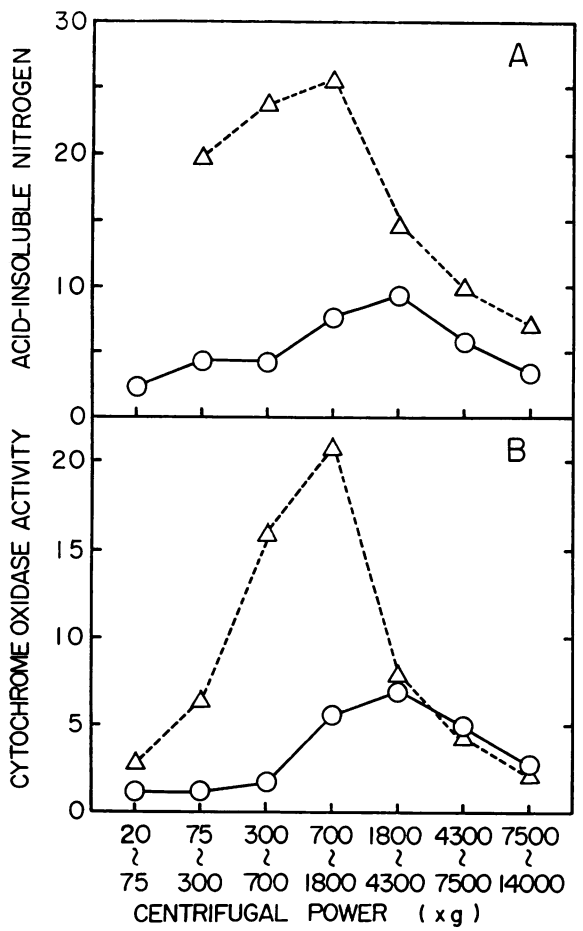

FIG. 1. Acid-insoluble nitrogen content and cytochrome oxidase activity in the various mitochondrial fractions precipitated by the centrifugation at different speeds. The homogenate prepared as described in Materials and Methods was centrifuged at 20 × *g* for 10 minutes and the 20 to 75 fraction was collected by centrifuging the supernatant fraction at 75 × *g* for 10 minutes. The 75 to 300 fraction was collected by centrifuging the supernatant fraction, which was obtained by the centrifugation at 75 × *g* for 10 minutes, at 300 × *g* for 10 minutes. Other fractions were collected by the same procedures. All fractions were finally washed with 0.05 M Tris buffer (pH 7.0) containing 0.5 M sucrose and suspended in 0.01 M phosphate buffer (pH 7.0). A) acid-insoluble nitrogen content in the various fractions in μg of nitrogen per g of fresh weight of tissue. B) cytochrome oxidase activity in the fractions in mμmoles of O_2 uptake per minute per g of fresh weight of tissue. —○—○—: fractions from the fresh tissue. —△—△—: fractions from the tissue aged for 2 days after slicing.

medium per definite weight of the tissue did not result in an increase of acid-insoluble nitrogen content or cytochrome oxidase activity in the mitochondrial fraction prepared from the fresh tissue (table II). In case of the tissue aged for 2 days after slicing, recoveries of mitochondrial fraction were higher when homogenization was done in 5 volumes instead of 2 volumes of medium per g of tissue. In general, the specific activity of cytochrome oxidase was found to decrease in the both fresh and wounded tissues when larger volumes of the medium was used.

In order to obtain quantitative yields of the mitochondrial fractions, repeated extraction of the same lot of tissue was attempted. Table III shows that the total acid-insoluble nitrogen content and cytochrome oxidase activity of mitochondrial fractions from 4 extractions of fresh tissue was less than that obtained by similar extraction procedures from tissue incubated for 2 days after slicing. The data in table III also show that the first extraction recovers over 70 % of the total nitrogen and nearly 80 % of the total cytochrome oxidase activity obtained from fresh tissue by 4 extractions. Similar results were obtained with wounded tissue.

Fractionation of Mitochondrial Particles by Differential Centrifugation. The mitochondrial fraction was separated into various fractions by centrifugation. Figure 1 shows acid-insoluble nitrogen

content and cytochrome oxidase activity in the different fractions. Cytochrome oxidase activity patterns suggest that most of the mitochondrial particles were precipitated between 600 and 14,000 × *g* in case of the fresh tissue, and between 75 and 4,300 × *g* in case of the wounded tissue. Only in a few experiments the precipitating pattern of mitochondrial particles from the wounded tissue was observed to be identical to that in case of the fresh tissue.

Table IV. *Size of Mitochondrial Particles*

The heavy and light fractions were obtained by collecting the particles precipitating between 100 and 2,000 × *g* and between 2,000 and 13,500 × *g*, respectively. The size was determined by measuring the diameter of the particles from photographs taken by electron microscopy at 5,000-fold magnification. The values show the length of the long axis. The values in parentheses show the standard deviation between 50 particles.

Time after slicing (days)	Size: Heavy fraction (μ)	Size: Light fraction (μ)
0	1.45 (0.33)	1.87 (0.48)
1	1.30 (0.21)	1.41 (0.29)
2	1.31 (0.23)	1.49 (0.32)

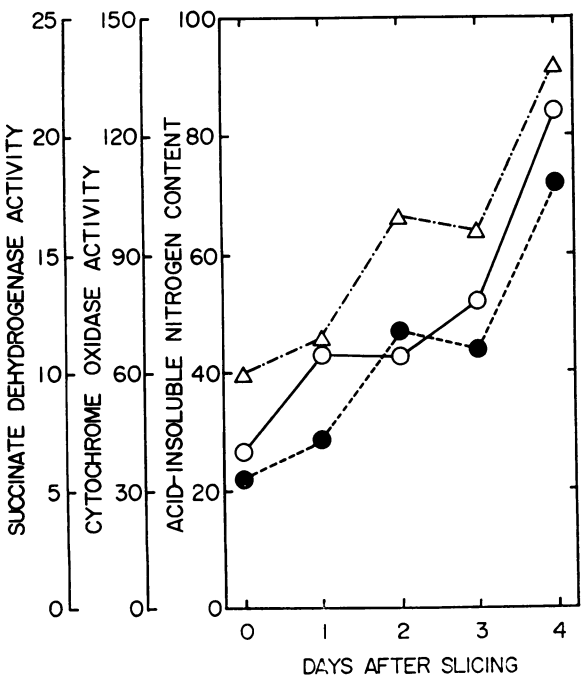

FIG. 2. Acid-insoluble nitrogen content, cytochrome oxidase activity and succinate dehydrogenase activity in the mitochondrial fraction as a function of time after slicing. —○—○—: the nitrogen content in μg of nitrogen per g of fresh weight of tissue. —●—●—: cytochrome oxidase activity in mμmoles of O_2 uptake per g of fresh weight of tissue. —△—△—: succinate dehydrogenase activity in decrease in optical density at 400 mμ per 10^3 minute per g of fresh weight of tissue.

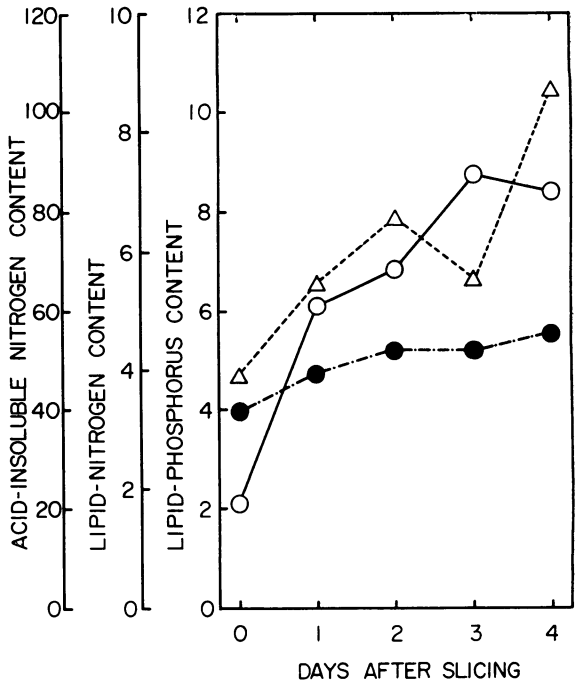

FIG. 3. The increase of lipid-phosphorus and -nitrogen contents in the mitochondrial fraction during the aging of slices of sweet potato root tissue. —△—△—: lipid-phosphorus content in the mitochondrial fraction in μg of phosphorus per g of fresh weight of tissue. —●—●—: lipid-nitrogen content in the mitochondrial fraction in μg of nitrogen per g of fresh weight of tissue. —○—○—: acid-insoluble nitrogen content in the mitochondrial fraction in μg of nitrogen per g of fresh weight of tissue.

However, the electron microscopic observations suggest that the particles precipitated by a low centrifugal power were not larger than those by a higher centrifugal power (table IV). No detectable difference in the size of mitochondrial particles from the fresh and wounded tissues was observed.

Increase of Mitochondrial Fraction during Aging of Slices. Figure 2 shows that increased amounts of the mitochondrial fraction can be extracted from slices of sweet potato root tissue during aging. Succinate dehydrogenase activity as well as acid-insoluble nitrogen content and cytochrome oxidase activity in the mitochondrial fraction were found to increase during aging.

Figure 3 shows that lipid-phosphorus and -nitrogen content of the mitochondrial fraction also increased during aging of the slices. The pattern of increase in lipid content was almost the same as that of the enzymic activities.

Increase in Number of Mitochondrial Particles in Tissue during Aging of Slices. Table V shows the increase in numbers of mitochondrial particles

Table V. *Increase in Number of Mitochondrial Particles during Aging of Slices of Sweet Potato Root Tissue*
The values show the percentage of the nitrogen content, the enzymic activity and the number of Janus green B-stained particles in the mitochondrial fraction from a given tissue relative to that obtained from fresh tissue. The values in parentheses indicate the standard deviation between 20 fields of microscopic vision.

Time after slicing (days)	Experiment I			Experiment II		
	Acid-insoluble nitrogen (%)	Cytochrome oxidase activity (%)	Relative no. (%)	Acid-insoluble nitrogen (%)	Cytochrome oxidase activity (%)	Relative no. (%)
0	100	100	100 (22)	100	100	100 (23)
1	...	...	...	151	142	112 (39)
2	193	270	150 (32)	191	359	173 (60)
3	201	318	278 (62)	189	355	238 (69)

during aging of the slices. It is evident that the Janus green B-stained particles in the mitochondrial fraction increased after 3 days. Marked increase in acid-insoluble nitrogen content and cytochrome oxidase activity also occurred. The standard deviation between fields of microscopic vision is too great to permit drawing a relationship between the increase in the nitrogen content and enzymic activity and in the number of the particles. It was observed that the number of the particles seemed to increase most rapidly between the second and third days after slicing of the tissue. However, the nitrogen content and the enzymic activity in the mitochondrial fraction increased more rapidly during the first 2 days after slicing.

Discussion

It was observed that the mitochondrial fraction in slices of sweet potato root was increased by aging or inoculation with *Ceratocystis fimbriata*. Verleur and Uritani (11) indicated that the increase in mitochondrial nitrogen in white potato tubers after cutting but not after infection with *Ceratocystis fimbriata*. In the present studies the increase was observed even in the inner tissue at the distance of 3 to 4 mm from the cut or infected surface. This result indicates that the increase in the mitochondrial fraction was not due to an increase in the number of the cells. Thus, it is proposed that the mitochondrial fraction in a cell increased during the aging of the root slices.

In the isolation of the mitochondrial fraction from the fresh tissue, increasing the volume of the homogenizing medium per the tissue weight did not increase the yield of acid-insoluble nitrogen or cytochrome oxidase activity. The recovery of the mitochondrial fraction from the wounded tissue was similar to that obtained with fresh tissue and was slightly enhanced by using a larger volume of the medium. Moreover, the yields of total nitrogen and cytochrome oxidase activity obtained by repeated extraction of wounded tissue was greater than that recovered from the fresh tissue. These results suggest that the observed increase in mitochondria was not due to a difference in the rate of extraction of the mitochondria from the 2 types of tissues.

The number of Janus green B-stained particles in the mitochondrial fraction was observed to increase in response to the slicing of the tissue. Thus, the increase in the acid-insoluble nitrogen content, the lipid content and the activities of respiratory enzymes in the mitochondrial fraction is considered to be the result of the biogenesis of mitochondria in the cells, in addition to the increase in these components of each mitochondrion.

There appears to be some difference in the properties of mitochondria from the fresh and wounded tissues. The difference was observed in the pattern of precipitation during centrifugation. Moreover, there also were different patterns of increases in the acid-insoluble nitrogen, the lipid content, the activities of respiratory enzymes, and the number of Janus green B-stained particles in the mitochondrial fraction during the aging of the slices. The increase in the nitrogen content appeared to precede to the increase in the enzymic activity, lipid content and number of the particles. However, these quantitative relationships should be investigated more in detail, after confirming that other particles such as the nuclei and ribosomes did not contaminate the mitochondrial fraction.

Acknowledgment

The authors express their thanks to Professor A. Hirai of Laboratory of Plant Pathology, Faculty of Agriculture, Nagoya University for his advices in the staining of mitochondrial particles and to Dr. T. Matsui of the same laboratory for taking the electron microscopic photographs. They are also very grateful to Dr. T. Kosuge of Department of Plant Pathology, University of California, Davis, California for his cordial assistance in preparing this manuscript. They also thank Research Laboratories of Sankyo Company Ltd. for the gift of cytochrome c.

Literature Cited

1. AKAZAWA, T. AND I. URITANI. 1956. Respiratory increase and phosphorus and nitrogen metabolism in sweet potato infected with *Ceratocystis fimbriata*. J. Biochem. (Japan) 43: 579–87.
2. BONNER, W. D. 1955. Succinic dehydrogenase. In: Methods in Enzymology. S. P. Colowick and N. O. Kaplan, ed. Academic Press, New York. Vol. I. p 722–29.
3. CLICK R. E. AND D. P. HACKETT. 1963. The role of protein and nucleic acid synthesis in the development of respiration in potato tuber slices. Proc. Natl. Acad. Sci. U. S. 50: 243–50.
4. FOLCH, J., M. LEES, AND G. H. S. STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226: 497–509.
5. HACKETT, D. P., D. W. HAAS, S. K. GRIFFITHS, AND D. J. NIEDERPRUEM. 1963. Studies on development of cyanide-resistant respiration in potato tuber slices. Plant Physiol. 35: 8–19.
6. HAGIHARA, B. 1961. Techniques for the application of polarography to mitochondrial respiration. Biochim. Biophys. Acta 46: 134–42.
7. JOHNSON, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137: 575–86.
8. THIMANN, K. V., C. S. YOCUM, AND D. P. HACKETT. 1954. Terminal oxidase and growth in plant tissue. III. Terminal oxidation in potato tuber tissue. Arch. Biochem. Biophys. 53: 239–57.
9. URITANI, I. 1963. The biochemical basis of disease resistance induced by infection. In: Perspectives of Biochemical Plant Pathology. The Symposia and Lectures Commemorating the 75th Anniversary of the Department of Plant Pathology and Botany. New Haven, Connecticut. p 4–14.
10. VERLEUR, J. D. AND I. URITANI. 1965. Mitochondrial activity in healthy white potato tubers and in potatoes infected with *Ceratocystis fimbriata*. Agr. Biol. Chem. 29: 369–71.
11. VERLEUR, J. D. AND I. URITANI. 1965. Respiratory activity of the mitochondrial fractions isolated from healthy potato tubers and from tuber tissue incubated after cutting or infection with *Ceratocystis fimbriata*. Plant Physiol. 40: 1008–12.