Respiratory Activity of the Mitochondrial Fractions Isolated from Healthy Potato Tubers and from Tuber Tissue Incubated after Cutting or Infection with Ceratocystis fimbriata[1]

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The respiratory activity of many plant tissues is considerably increased after infection with microorganisms. Initially an uncoupling of the respiration from the oxidative phosphorylation was thought to be responsible for this effect. Since then ample evidence has been accumulated which suggests that a metabolic activation leading to an accelerated utilization of high energy phosphate induced the increase of the O_2 uptake after infection (1, 11).

The respiratory increase occurring in potato tuber tissue after infection with various fungi (2, 12) or after wounding (4, 7, 8) has also been explained on the basis of the continued coupling between oxidative and phosphorylative processes. However, all evidence has been of a more or less indirect nature, since it was gathered from experiments with uncoupling agents and from results indicating an increase of the synthetic activity in the tissues.

Bonner and Voss (3) were the first who described respiratory control by ADP in isolated plant mitochondria. Wiskich and Bonner (13) isolated mitochondria from potato tuber tissue in which the rate of O_2 uptake was accelerated by addition of ADP but decreased again when the ADP had been consumed. In both investigations the oxygen electrode method (5) was used for the study of mitochondrial activity. However, the mitochondria isolated by Wiskich and Bonner as well as those described by Hackett et al. (4) showed phenomena which suggested that the particles might have been damaged during isolation.

The present paper deals with experiments with mitochondria isolated by a somewhat changed method resulting in particulate suspensions in which the mitochondria seem to be in a better condition than those previously described (Verleur, 1965, Plant Physiol. In press).

Materials and Methods

Preparation of Tissue Samples. Potato tubers (*Solanum tuberosum* L., var. Norin 1) were stored at 10°. Sound tubers were brushed in tapwater, externally sterilized in 4 % formalin for 2 minutes, washed in running tapwater for 15 minutes, and cut into 1-cm thick slices. These slices were placed in glass jars either after inoculation of the cut surface with a spore suspension of *Ceratocystis fimbriata* or without inoculation, and incubated at 25°. In some experiments 3-mm thick slices were incubated without infection.

Tissue samples were taken at 24-hour intervals. From the inoculated slices the apparently noninvaded tissue (zone of 1.5 mm thickness) adjacent to the invaded parts was collected (infected tissue). Usually the corresponding layers at 0.5 to 2 mm from the surface were taken from the 1-cm thick uninfected slices, but in some experiments the surface layers have been included in the samples (tissue 0–1.5 mm from the surface). The 3-mm thick slices were homogenized as a whole.

Only tissue from the central parts of the tubers has been used, the peel layers being removed just before sampling.

Isolation of Mitochondria. Tissue samples of 65 g were homogenized in a Waring blendor with 250 ml medium containing 0.7 M mannitol, 0.01 M K-phosphate buffer pH 6.5, 0.001 M EDTA, 0.002 M cysteine, and 0.1 % (w/v) BSA (bovine serum albumin, Armour Pharmaceutical Company, fraction V), pressed through a double layer of cheese cloth and centrifuged at 100 × *g* for 10 minutes (Verleur, 1965, Plant Physiol. In press). The supernatant fluid was centrifuged at 8000 × *g* for 10 minutes and the sediment washed twice with a mannitol-phosphate buffer-BSA medium without EDTA, and containing 0.001 M cysteine only during the first washing. The precipitate obtained after the final centrifugation at 8000 × *g* for 10 minutes was resuspended in a known volume of 0.7 M mannitol, 0.01 M phosphate buffer pH 6.5 and 0.1 % BSA, yielding the mitochondrial suspension for the experiments.

Respiration of Mitochondria. The O_2 uptake of these mitochondrial suspensions was recorded at 25° with succinate as the substrate using the oxygen electrode method (5). The reaction medium (3.2 ml containing 0.7 M mannitol, 0.01 M K-phosphate buffer pH 6.5, 0.01 M Tris pH 6.5, 0.0005 M EDTA, and 0.1 % BSA) and 0.4 ml mitochondrial suspension were brought into the vessel, after which 10 μmoles succinate and later 0.5 μmole ADP were added. The final volume of the reaction mixture was 3.7 ml.

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In the experiments in which the superficial layers have been removed before sampling an effect of bacterial contamination on the respiration of the mitochondrial preparations can be excluded. In those cases where the surface of noninfected slices was part of the samples, such an effect can be neglected, because the surface dried and a cork layer was formed during incubation. Only seldom a slight local bacterial growth was observed on very few slices after prolonged incubation (more than 1 week).

Calculation of Respiratory Control Ratio and ADP/O Ratio. The rates of O_2 uptake during the 4 states of reaction were determined from the oxygen electrode records. The respiratory control (R.C.) ratios have been calculated as O_2 uptake per minute during state 3/O_2 uptake per minute during state 4. The ADP/O ratios represent the quotients of the amount of ADP added to the reaction mixture (0.5 μmole) and the total O_2 uptake (μatom O) during state 3.

Nitrogen Content and Cytochrome Oxidase Activity. The nitrogen content of the mitochondrial suspensions has been determined by using Nessler's reagent after digestion in concentrated H_2SO_4 (9) and has been corrected for the nitrogen present in an equal volume of the suspension medium.

Cytochrome oxidase activity was determined in the oxygen electrode by measuring the O_2 uptake of a mixture of mitochondria, 0.04 M phosphate buffer pH 7.0, 3×10^{-5} M cytochrome c and 8×10^{-3} M Na-isoascorbate in a total volume of 3.7 ml. The enzyme activity was assayed at 30° using 3 different dilutions of each mitochondrial suspension in the range in which the O_2 uptake proved to be proportional to the amount of mitochondria in the reaction mixture (less than 100 γ mitochondrial nitrogen, maximal 10 mμmoles O_2 uptake per min).

Results

Results obtained with the oxygen electrode assay with mitochondria from healthy and infected tuber tissue are shown in figure 1. An ATP induction of the ADP effect (13) is obviously absent. Although not represented in the figure, it has been observed that an addition of ATP does not change the rate of succinate oxidation in the absence or presence of ADP.

All mitochondrial preparations clearly exhibit the phenomenon of respiratory control by ADP, including the decrease of the O_2 uptake after the depletion of ADP (state 4). The respiratory control (R.C.) ratios are 3.0 to 3.7 for mitochondria from healthy intact tubers stored at 10° (fig 1 A) and 2.0 to 2.6 in case of infected tissue (fig 1 C, D). However, the infected tissue and the healthy intact tubers stored at 10° have been treated in a different way. The infected tissue has been incubated at 25° after slicing of the tubers and after inoculation. Therefore, in addition, mitochondria have been prepared from

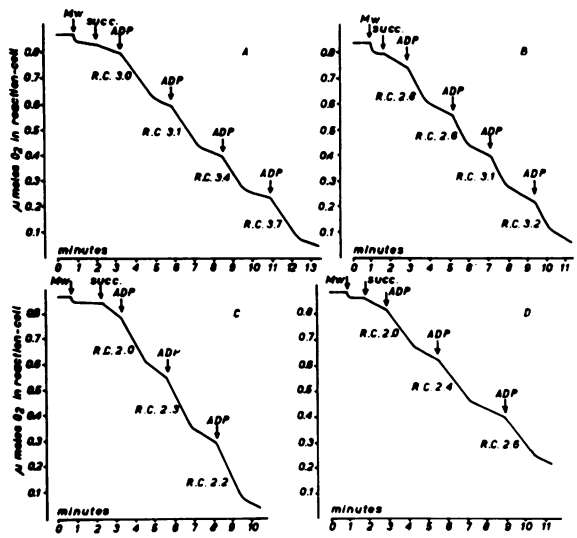

FIG. 1. Respiration of mitochondria from healthy potato tubers and from tuber tissue infected with *Ceratocystis fimbriata* as recorded by the oxygen electrode method. A) Tissue from healthy intact tubers stored at 10°. B) Tissue from healthy intact tubers incubated at 25° for 2 days. C and D) Tissue from infected tuber slices incubated at 25° for 2 and 3 days, respectively. Mw: 0.4 ml mitochondrial suspension. succ: succinate 10 μmoles per reaction. ADP: 0.5 μmole per reaction. R.C.: respiratory control ratio (O_2 uptake state 3/O_2 uptake state 4).

healthy intact tubers which have been incubated at 25° for 2 days (fig 1 B). The R.C. ratios prove to be of the same order (2.6–3.2) as those found with tubers stored at 10°, and they are clearly higher than the R.C. ratios of infected tissue mitochondria.

In order to obtain samples of infected tissue, the tubers had to be sliced preceding inoculation and incubation. Thus, it was necessary to determine if slicing of the tubers affected the respiratory control of the isolated mitochondria. In all experiments the succinate oxidation of preparations from cut tissue proved to be controlled by ADP (table I). The R.C. ratios of the particulate fractions from the cut tissue corresponding to the layers taken from inoculated slices (0.5–2 mm from the surface) are lower than the ratios observed using intact tubers. They are of the same order as found with infected tissue. When surface layers of the slices are included in the samples (0–1.5 mm from the surface) the ratios of the resulting preparations seem to have a tendency to decrease further, at least when the slices have been incubated for 3 days. This tendency is more clear when tuber slices of 3-mm thickness are used for the isolation of particles, especially after 3 days of incubation. It should be noticed that 3-mm slices consist of 2 zones of tissue at 0 to 1.5 mm from the surface.

Although generally the rate of succinate oxidation has been increased after infection or cutting, it is

Table I. *Properties of Mitochondria from Healthy Potato Tubers and from Tuber Tissue Infected with Ceratocystis Fimbriata or Incubated after Cutting without Infection*

The data are averaged for 3 experiment series.

	R.C.*	ADP/O**	γ N***	O_2 uptake† State 3	O_2 uptake† State 4	O_2 uptake/ γ N State 3	Cytochrome† oxidase	Cytochrome oxidase per γ N
Healthy tubers stored at 10°	3.0	1.7	342	132	44	0.4	144	0.4
Healthy tubers incubated at 25° for 2 days	3.0	1.7	412	134	45	0.3	245	0.6
Infected tissue incubated at 25° for 2 days	2.2	1.3	406	165	75	0.4	236	0.6
3 days	2.1	1.2	316	135	64	0.4	193	0.6
Cut tissue: 0.5–2 mm from surface of 1-cm slices incubated for 1 day	2.5	1.2	495	121	48	0.3	258	0.5
2 days	2.2	1.3	454	172	78	0.4	260	0.6
3 days	2.3	1.3	593	178	77	0.3	346	0.6
Cut tissue: 0–1.5 mm from surface of 1-cm slices incubated for 2 days	2.2	1.2	492	174	79	0.4	359	0.7
3 days	2.0	1.1	568	322	161	0.6	368	0.7
Cut tissue incubated as 3 mm-thick slices for 2 days	2.0	1.0	566	187	93	0.3	377	0.7
3 days	1.5	0.8	646	170	113	0.3	404	0.6

* R.C.: respiratory control ratio (O_2 uptake per min state 3/O_2 uptake per min state 4).

** ADP/O: amount of ADP added (0.5 μmole) / total O_2 uptake in μatom O during state 3.

*** Nitrogen content of Mw suspension: γ nitrogen per 10 g original tissue (corrected for the nitrogen present in the suspension medium).

† O_2 uptake during state 3 and state 4 cytochrome c oxidase activity — mμmoles O_2 per minute per 10 g original tissue.

evident from table I that the activity during state 4 increases to a relatively greater extent than the oxidation during state 3 does, causing the fall of the R.C. ratios.

The increase in activity of the preparations from cut tissue is greater both during state 3 and state 4, when the surface layers have been included in the samples, although there seems to exist some difference in behaviour between the fractions from tissue incubated as 3-mm slices and those from 1-cm slices. Special attention has to be paid to the observation that in contrast to mitochondrial fractions prepared from infected tissue after 2 days of incubation, which have a higher oxidative activity comparable with the respiration rate of particles from the corresponding layers of cut tissue, in the fractions isolated 3 days after inoculation the O_2 uptake during state 3 has already fallen to the level of the healthy controls. The O_2 uptake during state 4 decreased to a lesser extent.

The oxidative activity of the mitochondrial fractions during state 3 is higher after infection and after cutting. This might be due to an increase in the amount of mitochondria or to a higher activity of the particles. Therefore, the nitrogen content and the cytochrome oxidase activity in the fractions have been determined, in order to know whether the amount of mitochondria changes or not.

The mitochondrial fraction from 2 days incubated infected tissue contains more nitrogen than the preparations from healthy intact tubers stored at 10° (table I). However, the nitrogen content appears to be equal to that of the fraction isolated from healthy intact tubers which have been incubated at 25° for the same period. In accordance with the observed fall in oxidative activity in the preparations from 3 days incubated infected tissue, the nitrogen content of these fractions is also lower than after 2 days of incubation.

Tuber slices incubated without inoculation always yielded higher amounts of nitrogen in the isolated fractions compared with the tissue from both healthy tubers stored at 10° and tubers incubated at 25°. The nitrogen content is even higher after cutting than after infection. Especially when the cut tissue has been incubated as 3-mm thick slices, the increase in nitrogen content is striking and seems to take place sooner after cutting than in slices of 1-cm thickness. Calculation of the rate of O_2 uptake during state 3 per γ nitrogen makes it clear that practically no change in oxidative activity per unit of nitrogen occurs.

The data of table I also clearly demonstrate that in general the cytochrome oxidase activities of the isolated fractions follow the same pattern as the nitrogen content does. This is more evident when

the cytochrome oxidase activity is calculated per γ nitrogen present in the preparations. With the exception of the healthy intact tubers stored at 10°, which gave a somewhat lower value, all tissues yielded mitochondrial fraction with practically the same cytochrome oxidase activity per unit of nitrogen.

Discussion

The R.C. ratios of the mitochondrial respiration observed in preparations from healthy tissue are higher than those reported for potato tuber mitochondria by Wiskich and Bonner (13). The occurrence of a clear respiratory control by ADP in mitochondria from both noninfected tissue and infected tissue demonstrates in a direct way that also after infection the oxidative and phosphorylative processes are coupled.

However, the R.C. ratios and ADP/O ratios of mitochondria from both infected tissue and cut tissues are lower than in the healthy controls. These lower ratios are due to the higher rate of O_2 uptake during state 4, which has been accelerated to a greater extent than the O_2 uptake during state 3. This might be explained by the assumption that respiration and oxidative phosphorylation in the infected and cut tissues are partially uncoupled. However, in the authors' opinion, another way of interpretation of the results may be more likely. An increase of the adenosine triphosphatase activity in mitochondrial preparations with maintenance of a complete coupling between oxidation and phosphorylation will also result in a rate of O_2 uptake which is relatively more increased during state 4 than during state 3, and consequently lead to lower R.C. and ADP/O ratios. This might be induced by changes in physical properties of the mitochondria in the cells as a result of the marked metabolic activation, or by damaging effects during the isolation procedure of compounds (e.g. phenolic compounds) accumulating in the tissue during incubation (6, 10).

The R.C. and ADP/O ratios are reduced more in mitochondria from 3-mm thick slices than in mitochondria from 1-cm thick slices. In other experiments (12) an acceleration of the O_2 uptake following cutting of potato tubers could be demonstrated as far as at least 2 mm from the cut surface. When 1-cm thick slices are incubated, these zones of 2 to 3 mm from both surfaces are separated by cell layers, while in 3-mm slices these 2 zones are overlapping. Consequently, the activating effect of the cutting on cell metabolism and/or accumulation of compounds can be expected to be much more intensive in 3-mm slices. The greater and earlier increase of the nitrogen content and the cytochrome oxidase activity of the mitochondrial fractions from these 3-mm thick slices might be explained on this basis. If the hypothesis is right that this intensive wound reaction affects the properties of the mitochondria in the tissue and makes them suffer more injury during the extraction, then the low R.C. and ADP/O ratios are understandable.

It has been concluded from table I that all tissues yielded mitochondrial fractions with practically the same O_2 uptake during state 3 and cytochrome oxidase activity per unit of nitrogen. This strongly suggests that both the changes in nitrogen content and in cytochrome oxidase activity represent changes in the amount of mitochondria present in the fractions without marked changes in activity of the mitochondria.

The nitrogen content and cytochrome oxidase activity of the mitochondrial suspensions from cut tissue are clearly higher compared with preparations from healthy intact tubers incubated at 25°, indicating an increase in mitochondrial number after cutting. However, such an increase has not been observed in the fractions from infected tissue, which even show a decrease 3 days after inoculation. This phenomenon might be explained on the assumption that 3 days after inoculation a degeneration of mitochondria has occurred in the infected tissue. Perhaps, this degeneration starts earlier, resulting in the fact that in contrast to the fractions from the cut tissues, those from 2 days incubated infected tissue do not show an increase in the amount of mitochondria. However, the equal R.C. and ADP/O ratios 2 and 3 days after infection makes a degeneration of mitochondria in the tissue dubious, the more so as it is known that a respiratory increase after infection can be demonstrated in potato tuber tissue adjacent to the invaded parts after much longer incubation periods (12). Therefore, the decrease in the amount of mitochondria in the isolated fractions might be an indication for the possibility that it is more difficult to extract mitochondria from infected tissue than it is from noninfected tissues.

Summary

Mitochondria have been prepared from intact potato tubers (*Solanum tuberosum* L.), from tuber slices (cut tissue) and from the tissue adjacent to the invaded parts of 1-cm thick slices inoculated with *Ceratocystis fimbriata* (infected tissue). All preparations showed respiratory control by ADP; the oxidative and phosphorylative processes were coupled, also after infection.

The R.C. and ADP/O ratios of mitochondria from infected and cut tissues proved to be lower than in mitochondria from healthy tubers.

The changes in nitrogen content and cytochrome oxidase activity in the mitochondria ran in parallel, indicating changes in the amount of mitochondria. The cytochrome oxidase activity per γ nitrogen was practically the same in all preparations.

The experimental results suggest that the amount of mitochondria in cut tissue was higher than in intact tubers. No increase in mitochondrial number was found in preparations from infected tissue when

compared with the fractions from incubated intact tubers. Even a decrease was observed 3 days after inoculation.

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