Studies on the Isolation of Mitochondria from Potato Tuber Tissue

J. D. Verleur

Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Anjo, Aichi, Japan

During the last decade many reports on the isolation from plant tissues of particulate fractions containing many respiratory enzymes, have been published (5). These fractions proved to be very suitable for a detailed study of respiratory metabolism, the more so because plant mitochondria which after addition of substrate and cofactors gave O2 uptake coupled with phosphorylating processes, could be isolated.

The effect of 2,4-dinitrophenol upon the respiration rate of potato tuber tissue (15) strongly suggests that under normal conditions respiratory activity and oxidative phosphorylation are coupled, and regulated by the availability of phosphate acceptor (ADP). Therefore, also after isolation, the mitochondria should have a substrate oxidation which is coupled with phosphorylation, and the rate of which is controlled by ADP.

Hackett et al. (8) were the first who described the ability of potato tuber mitochondria to carry out oxidative phosphorylation. In their experiments using the Warburg respirometer and using succinate as the substrate they found P/O ratios of around 1.3, but they did not obtain evidence for a respiratory control by ADP.

The best method available for investigating respiratory control in mitochondria is the oxygen electrode method (9) which allows short-term experiments. Bonner and Voss (2) using this highly sensitive method were the first who succeeded in demonstrating a respiratory control by ADP in isolated plant mitochondria. Later Wiskich and Bonner (17) were able to prepare mitochondrial fractions from potato tuber tissue, which not only showed accelerated O2 uptake upon addition of ADP, but also a decreased rate of substrate oxidation when the ADP had been consumed. They found respiratory control ratios (R.C. ratios) of around 2.0.

Although Hackett et al. (8) and Wiskich and Bonner (17) made great progress in isolating mitochondria from potato tuber tissue, their preparations had some properties which suggested that the condition of the particles might not have been very good. Wiskich and Bonner could already eliminate the marked increase of the respiration upon addition of cytochrome c, as described by Hackett et al. (8), but their preparations exhibited a considerable induction period for the ADP stimulation of the succinate oxidation. Such an induction period could be avoided by preincubation of the mitochondria with ATP. Now it is known that ATP has an effect on the maintenance of contraction in mitochondria (7, 13). So the ATP induction phenomenon may be an indication that the mitochondria require a recovery, induced by ATP, from changes occurring during the isolation procedure, before they can react to addition of ADP.

Therefore, the conditions for the isolation of mitochondria from potato tuber tissue have been investigated in order to obtain mitochondria in a better condition than described up until now.

Materials and Methods

Potato tubers (Solanum tuberosum L., var. Norin 1) purchased from the local market were stored at 10°C. Intact tubers were refrigerated in the cold room (4°C) for at least 1 hour. After peeling, tissue blocks of less than 0.5 cm3 were cut from the central parts of the tubers and immediately used for the isolation of mitochondria. The media for the isolation were prepared with glass-distilled water immediately before use, and prechilled in ice for at least 1 hour. All operations were carried out at roughly 0°C.

Preparation of Mitochondrial Fractions I. To minimize the disorganization of the mitochondria themselves, the tissue was homogenized for only 30 seconds in a Waring blender alternately at low speed for 3 seconds and at high speed for 2 seconds. Preliminary observations showed that blending for 30 seconds or longer at high speed did not disintegrate the tissue to a much greater extent and yielded mitochondria which were in bad condition.

The media used were based on the methods described by Wiskich and Bonner (17) and Hackett et al. (8). Tissue samples of 150 g were homogenized in 300 ml medium containing 0.5 M mannitol, 0.05 M K-phosphate buffer pH 7.2, 0.01 M EDTA, 0.02 M cysteine and 0.1 % BSA (bovine serum albumin, Armour Pharmaceutical Company, fraction V), pressed through a double layer of cheese cloth and centrifuged at 800 × g for 10 minutes. The supernatant fluid was centrifuged at 8000 × g for 10

1 Received April 27, 1965.
2 Present address: Department of Botany, Free University, Amsterdam, The Netherlands.
minutes and the sediment washed twice with 0.5 M mannitol in 0.05 M K-phosphate buffer medium pH 7.2, containing during the first washing 0.01 M cysteine and 0.1 % BSA, and during the second washing 0.005 M cysteine without BSA. The precipitate obtained after the final centrifugation at 8000 × g for 10 minutes was resuspended in a known volume of 0.5 M mannitol in 0.05 M K-phosphate buffer pH 7.2, yielding the mitochondrial suspension for the experiments. The reaction medium was composed of 0.5 M mannitol, 0.02 M K-phosphate buffer pH 7.2, 0.01 M Tris pH 7.2, 0.0005 M EDTA and 0.005 M MgCl₂.

Preparation of Mitochondrial Fractions II. For reasons to be discussed below, the media used during the isolation procedure have been changed in order to obtain mitochondria in a better condition than after the preparation by using method I. The concentration of mannitol has been increased, those of K-phosphate buffer, cysteine and EDTA have been decreased, and the pH of all media and of the reaction mixture was lowered to 6.5. All media, including the reaction medium, contained 0.7 M mannitol, 0.01 M phosphate buffer and 0.1 % BSA. Cysteine was added to the homogenizing medium in a concentration of 0.002 M and to the first washing medium in a concentration of 0.001 M, whereas EDTA was added to the homogenizing medium and the reaction mixture in a concentration of 0.001 M and 0.0005 M, respectively. In addition 0.01 M Tris pH 6.5 was included in the reaction medium.

The isolating procedure, which was essentially the same as described for method I, is summarized in figure 1. Apart from the changes in the media there are 2 changes from method I viz. the increase of the volume of homogenizing medium per volume of tissue (nearly 4 ml per g of tissue) and the lower centrifuge speed during the first sedimentation (100 × g).

The Respiration of Mitochondria. For the estimation of the oxidative activity of the mitochondria 0.4 ml mitochondrial suspension and 3.2 ml reaction medium were brought into the vessel of the oxygen electrode (9), after which 10 μmoles succinate and later 0.5 μmole ADP were added. In some experiments 0.1 μmole ATP was given prior to the ADP. The final volume of the reaction mixture was 3.7 ml.

The O₂ uptake of the succinate oxidation was recorded at 25°. In the course of the oxygen electrode test, 4 states have been distinguished [see Chance and Williams (4)] viz. state 1 with only mitochondria added to the reaction medium, state 2 after subsequent addition of succinate, state 3 starting with the addition of ADP and terminating when the ADP had been consumed (as appears from the decreasing rate of O₂ uptake), and state 4 the period after depletion of the added ADP. The reaction changed back into state 3 when ADP was added once more.

The rates of O₂ uptake during the reaction were determined from the oxygen electrode records. The respiratory control (R.C.) ratios have been calculated by dividing the maximum rate of O₂ uptake in the presence of the added ADP by the rate when all ADP has been consumed. The ADP/O ratios represent the quotient of the amount of ADP added (0.5 μmole) and the total O₂ uptake (in μatom O) during state 3.

Results

Preparation Method I. A typical oxygen electrode curve obtained with mitochondria isolated by the first method is shown in figure 2. The low rate of O₂ uptake in the presence of succinate was markedly accelerated by addition of ADP, indicating a respiratory control by ADP. Just as in the experiments of Wiskich and Bonner (17), the acceleration of the O₂ uptake upon the first addition of ADP was rather slow and showed a certain lag phase. This lag phase was absent when ADP was given for a second time. In addition, the rate of substrate oxidation during state 4 was obviously higher than the rate during state 2. This indicated an activation during the reaction in the oxygen electrode of the processes which gave an oxidation either controlled by ADP and induced by phosphate acceptor regeneration (adenosine triphosphate activity), or not regulated by ADP. A calculation of reliable
R.C. ratios was impossible as the oxidation rates during states 2 and 4 were not equal.

Now it is known that Mg$^{2+}$ can stimulate the adenosine triphosphatase activity in mitochondrial preparations (14) and MgCl$_2$ was one of the components of the reaction mixture. So it seemed to be worthwhile to investigate the effect of the presence of Mg$^{2+}$. In the experiment shown in figure 3 samples of one isolation of mitochondria have been tested in both a reaction mixture containing MgCl$_2$ and in a mixture in which the MgCl$_2$ had been replaced by KCl. In the KCl medium no or hardly any increase occurred of the oxidation during state 4 compared with the rate during state 2. In the MgCl$_2$ medium the increase was again considerable. The mitochondria react to the first addition of ADP rather slowly in both media, but the lag phase seemed to be shorter in the KCl medium.

In contrast to the results described by Wiskich and Bonner (17) the lag phase did not disappear when ATP was added to the reaction preceding the ADP. The rate of O$_2$ uptake during state 2 was hardly or not accelerated by ATP. This was again in contrast to the results of Wiskich and Bonner who found that the rate of O$_2$ uptake of mitochondria with only succinate added was much lower than when the mitochondria had been preincubated with ATP, whereas in both cases the oxidation during state 3 reached roughly the same speed.

In conclusion, this way of isolation did not result in mitochondrial preparations which were in better condition than those described already in literature. Yet, the results strengthened the impression that the fractions described contained more or less damaged mitochondria, and the isolation procedure should be improved.

**Discussion**

*Results with Preparation Method II.* Several changes have been introduced in the preparation method described above.

**Homogenization.** During and after the drastic disorganization of the tissue certain cell constituents might affect the mitochondria before they have been separated from the homogenate. Homogenizing for a short time (30 sec) followed by a rapid separation limits the period of contact. In addition, the concentration of compounds in the homogenate can be lowered by blending the tissue samples in several times their volume of medium. As the extent of dilution with medium is limited by the necessity to obtain preparations with a suitable activity, henceforth 65 g of tissue was homogenized with 250 ml of medium.

**pH.** The pH of the media is said to be an extremely critical factor when isolating mitochondria (17). In the work with plant tissues a pH 7.0 to 7.5 has generally been used (1,3,8,16), a higher or lower pH having deleterious effects. Reinvestigation of the effect of pH on the mitochondrial behaviour after isolation resulted in the observation that the best results could be obtained with media of pH 6.5 (table I).

**Mannitol.** Generally 0.5 m sucrose or mannitol was used in the isolation of plant mitochondria (1,3,8). Only Wiskich and Bonner (17) applied a total concentration of 0.62 m composed of 0.25 m sucrose and 0.37 m mannitol. Now, experiments on the effect of toxicity showed that it was possible to get reasonable mitochondria with media containing 0.5 to 0.7 m mannitol, although 0.7 m yielded the best preparations. The toxicity of the media proved to be less critical than has been claimed (8).
Table 1. The Effect of pH of the Isolation Media (Method II) on the \( O_2 \) Uptake of Potato Mitochondria

<table>
<thead>
<tr>
<th>pH</th>
<th>State 2</th>
<th>( O_2 ) Uptake State 3</th>
<th>State 4</th>
<th>Respiratory control ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>32</td>
<td>81-104</td>
<td>34</td>
<td>2.4-3.1</td>
</tr>
<tr>
<td>6.5</td>
<td>30</td>
<td>96-112</td>
<td>30</td>
<td>3.2-3.7</td>
</tr>
<tr>
<td>6.0</td>
<td>34</td>
<td>89-102</td>
<td>34</td>
<td>2.6-3.0</td>
</tr>
</tbody>
</table>

Cysteine. Cell constituents might affect the particles during isolation. Especially oxidation products of phenolic compounds proved to be harmful to plant mitochondria (10). Cysteine can prevent the browning of chlorogenic acid in the presence of polyphenol oxidase when present in a molar concentration greater than that of chlorogenic acid (16). However, cysteine also caused high rates of endogenous oxidation in sweet potato mitochondria (8).

In the inner tissue of potato tubers the concentration of phenolic compounds is much lower than in the peel layers (11,18). From data in the literature (11,12,18) it could be calculated that the concentration of the phenolics in the inner flesh is in the order of \( 10^{-3} \) to \( 10^{-4} \) M. As, in addition, these compounds are diluted by homogenizing the tissue in a greater volume of medium, the cysteine concentration in the homogenizing medium could be lowered to 0.002 M and still give prevention of browning of the homogenate. In order to use cysteine concentrations which are not higher than necessary, half this concentration was added to the first washing medium and cysteine was omitted from the other media.

Bovine Serum Albumin. Mitochondria from potato tuber tissue isolated using method I showed a better respiratory control by ADP when 0.1 % BSA (w/v) was added to the reaction mixture (6). In method II all media contained 0.1 % BSA, including the mitochondrial suspension and the reaction mixture in the oxygen electrode test.

EDTA. The swelling of rat liver mitochondria induced by cysteine was inhibited by EDTA, especially when EDTA was added simultaneously with cysteine (7). On the other hand, EDTA might remove too much of the catalytic metals. Therefore, it was added only to the homogenizing medium and the concentration was lowered to 0.001 M. The reaction mixture contained 0.0005 M.

Magnesium. Usually MgCl\(_2\) has been included in the reaction mixture for the study of respiratory properties of plant mitochondria. As shown in figure 3 the increase of the oxidation during state 4 compared with the rate during state 2, occurring when MgCl\(_2\) was present in the reaction mixture, was not found when it was replaced by KCl. In other experiments it proved to be possible to omit both MgCl\(_2\) and KCl from the mixture without any change in oxidative activity and respiratory control by ADP. Therefore neither MgCl\(_2\) nor KCl were added to the reaction mixture.

Centrifugation. Based on previous reports the mitochondria were originally separated as the fraction sedimenting between 8000 \( \times g \) and 8000 \( \times g \) (method I). However, results of Asahi and Honda in this laboratory (personal communication) indicated that after wounding sweet potato tissue the main peak of mitochondrial sedimentation changed to lower \( g \)-values. Therefore, in method II the mitochondrial fraction was collected between 100 \( \times g \) and 8000 \( \times g \).

All these considerations resulted in the modified isolation method described as method II and summarized in figure 1.

Using this method, mitochondrial fractions could be obtained from potato tuber tissue which gave a respiratory pattern as shown in figure 4.

![Figure 4: Respiratory pattern of mitochondrial fractions from potato tuber tissue](https://www.plantphysiol.org/)

The rate of \( O_2 \) uptake of the mitochondria with 10 \( \mu \)moles succinate was rather low and did not increase when 0.1 \( \mu \)mole ATP was added. The acceleration of the substrate oxidation upon addition of 0.5 \( \mu \)mole ADP was abrupt. The \( O_2 \) uptake immediately rose to a high, constant rate which was maintained till all ADP had been consumed and the reaction came into state 4. In the experiment of figure 4 ATP had been applied preceding the addition of ADP, but exactly the same results were obtained when ATP was not added.
The rates of $O_2$ uptake during state 4 after the subsequent additions of ADP turned out to be very constant and equal to the low rate during state 2 (mitochondria with succinate). There was no increase of state 4 activity during the reaction time.

The respiratory control ratios (R.C.) calculated from the oxygen electrode curve for the subsequent additions of ADP reached the value of 4.0, which was roughly twice the value of the R.C. ratio published for potato tuber mitochondria up till now (17). The ADP/O ratios were 1.4 to 1.7. Considering that the P/O ratio with succinate as the substrate has a theoretical value of 2.0, and that the ADP/O ratios were calculated without correction for the rate of oxidation during state 4. (Adenosine triphosphate?), the values for the ADP/O ratios were close to the theoretical value.

The immediate maximal response of the substrate oxidation to the addition of ADP without any sign of a lag phase, and the good respiratory control by ADP with R.C. ratios of 4.0 clearly demonstrate, in the author’s opinion, that the mitochondrial fractions separated using the modified method have been isolated in a better condition than described in literature.

The substrate oxidation was accelerated immediately after addition of ADP without any sign of a lag phase. It is concluded that the slow reaction to the addition of ADP found by Wiskich and Bonner was related to damage of the mitochondria in their preparations. With the modified procedure mitochondria could be isolated in a better condition.

**Summary**

A modified procedure is described for the isolation of mitochondria from potato tuber tissue. The respiratory behaviour of these particulate fractions was recorded in the oxygen electrode test with succinate as the substrate. The rate of $O_2$ uptake was controlled by ADP to a greater extent than in the preparations described in literature; the respiratory control ratios were roughly twice as high.

The substrate oxidation was accelerated immediately after addition of ADP without any sign of a lag phase. It is concluded that the slow reaction to the addition of ADP found by Wiskich and Bonner was related to damage of the mitochondria in their preparations. With the modified procedure mitochondria could be isolated in a better condition.

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**Literature Cited**


