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Influence of Ionizing Radiation on the Oxidative Phosphorylation, Adenosine Triphosphatase, and Apyrase in Potato Tubers

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The oxidative phosphorylation of isolated potato mitochondria has been studied. Preparations obtained from γ-irradiated tubers are compared to preparations from non-irradiated tubers. ATPase and apyrase activities in the mitochondrial, microsomal, and soluble fractions have also been investigated. Differences between oxidative phosphorylation in mitochondria from treated and from untreated tubers were observed only when the uncoupling agent dinitrophenol was present or when sub-optimal conditions prevailed. A minor increase was observed in the activity of apyrase isolated from irradiated tubers as compared to the corresponding enzyme from control tubers.

During the last decade our understanding of the respiratory electron transport has improved greatly. Most of the important advances have, however, been made with animal systems. To quote prominent authors in this field, the knowledge of the respiratory electron transport in plants is still in a relatively "backward state". Although oxidation and reduction of cytochromes in intact tissues of plants have been observed in numerous investigations (*e.g.* Refs. 1—3), detailed knowledge of the structure and function of the respiratory chain has come from studies on mitochondria and sub-mitochondrial fragments.

ATP is the most important carrier of metabolically available energy in cells. In mitochondria, multienzyme systems are involved which, through electron transport coupled oxidative phosphorylation, provide most of the ATP required for cellular function.[4,5] In the oxidative phosphorylation, electrons are sequentially transported from the substrates to oxygen by means of a chain of respiratory enzymes located in the mitochondria. This electron transport chain is coupled to a mechanism which forms ATP from ADP and orthophosphate.

To-day mitochondria comprise the standard starting material for examination of the respiratory electron transport chain.

Already ten years ago, Hackett [6] studied the pathways of oxidation in cell-free potato fractions. Hackett and co-workers [7] were the first to describe the ability of potato tuber mitochondria to carry out oxidative phosphorylation. Three years later, Wiskich and Bonner [8] described in detail how to isolate mitochondria with high enzyme activity from sweet potatoes (*Ipomea batatas*). These authors also isolated mitochondria from white potato tubers (*Solanum tuberosum*); they emphasized, however, that it is difficult to get "active" mitochondria from white potatoes (hereafter only called potatoes).

Verleur [9] recently investigated mitochondria isolated from potato tuber tissue using a modification of the method described by Wiskich and Bonner.[8] Verleur considered his mitochondria to be "in a better condition" than the preparations obtained by Wiskich and Bonner.

The present paper describes an attempt to isolate mitochondria from tubers of three different potato varieties. The aim was to investigate whether γ-irradiation of the tubers has any influence on the activity of the mitochondria with respect to the oxidative phosphorylation. Six months were spent in obtaining active mitochondria preparations. The technique used was a slight modification of the method described by Wiskich and Bonner.[8] The vital point seems to be the pH of the homogenisation medium and of the reaction medium.

Preliminary studies of crude preparations from potato tubers indicated that γ-doses of 14—15 kilorad caused a decreased activity of ATP-phosphohydrolase (E.C. 3.6.1.3) (ATPase) (unpublished results). In the present study, the activities of ATPase and ATP-diphosphohydrolase (E.C. 3.6.1.5) (apyrase) in the isolated mitochondria, in the microsomes, and in the soluble fraction were investigated. In addition, the activity of apyrase isolated from potato tubers according to Krishnan [10] and according to Cori *et al.*[11] was determined. The aim of this study was to compare enzymes (or fractions) originating from control tubers with corresponding enzymes or fractions from irradiated tubers.

MATERIALS AND METHODS

Three potato varieties commonly used in Sweden: Bintje, King Edward, and Early Puritan, were chosen for this investigation. The irradiation facilities have been described earlier,[12] the dose used was 14—15 kilorad and the dose rate 125—150 rad/sec. Irradiated tubers and non-irradiated control tubers were stored for at least one week at a temperature between +4° and +5°C and at 80 % RH (relative humidity) prior to use.

Preparation of mitochondria, microsomes, and the "soluble fraction". The tubers were rapidly peeled, 300 g cut into pieces and disintegrated in 600 ml of a medium containing 0.5 M mannitol, 5×10^{-3} M EDTA, 4×10^{-3} M cysteine and 0.1 % BSA (bovine serum albumin). The mixture was blended for 15 sec at low speed in a Waring Blendor; during the first seconds of blending, the pH was adjusted to the desired range by a rapid addition of 5.5 M KOH. The amount of KOH necessary for this pH had been determined in advance by taking samples of a similar homogenate and determining the pH in a pH-meter. Homogenates from irradiated and non-irradiated tubers required different amounts of KOH to attain the same pH. (Indicator paper does not give the correct pH as the medium contains EDTA).

The homogenate was squeezed through double gauze and the filtrate was centrifuged for 15 min at 800 *g* to remove starch and cell debris. The supernatant was centrifuged for 45 min at 10 000 *g*. The pellet obtained was washed once with 0.5 M mannitol including 0.1 % BSA and once with 0.5 M mannitol without BSA. By resuspending the pellet, the

concurrently precipitated small amount of starch could be carefully removed; after each washing the homogenate was centrifuged at 10 000 *g* for 45 min. The resulting pellet was suspended in 3.5 ml 0.5 M mannitol. All operations were carried out between 0° and +2°C. The mitochondria were used immediately after the isolation.

The supernatant from the isolation of the mitochondria was centrifuged for 50 min at 100 000 *g*. The microsomal pellet was washed in the same manner as the mitochondrial pellet, and the resulting pellet was suspended in 3.5 ml 0.5 M mannitol. The supernatant obtained is the "soluble fraction".

Measurement of oxygen uptake and oxidative phosphorylation of the mitochondria. The oxygen uptake was measured according to Warburg's method at +30°. The final concentrations of the components of the reaction medium were: 0.3 M sucrose, 0.01 M Tris-buffer pH 7.2, 0.03 M potassium phosphate, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.1 M glucose, 1 mM ATP and 1 mg hexokinase (Sigma, type III from yeast). 0.5 ml mitochondrial suspension (containing 0.70—1.50 mg N per ml) was used in each Warburg vessel and the final volume of the reaction mixture was 3 ml + 0.2 ml 20 % potassium hydroxide in the center well. The substrates used were succinate or malate (final concentrations 0.02 M) and NAD^+ or NADH (final concentrations 2×10^{-4} M). Where indicated, 2,4-dinitrophenol (DNP) was added to the reaction medium in a final concentration of 10^{-4} M. The medium was thermo-equilibrated for 10 min and the mitochondria were added from the side arm after 30 sec additional equilibration. The oxygen uptake was determined during 20 to 30 min depending on the activity of the preparation. The reaction was stopped by addition of 1 ml ice-cold 10 % trichloroacetic acid. After centrifugation of the samples, aliquots were taken for determination of the phosphate uptake in organic compounds; inorganic phosphorus was estimated by using three different methods: Fiske and Subbarow, modification Hahn,[13] Martin and Doty,[14] and Teorell.[15] As parallel determinations using the three methods all gave satisfactory results, and the method of Martin and Doty consumed a great deal of time, the method of Teorell was employed for the main part of the investigation. A determination of inorganic phosphorus initially present was carried out in the same way by introducing trichloroacetic acid before adding the other components, or by using water instead of the mitochondrial suspension. Mitochondrial respiratory control was determined by omitting hexokinase and glucose from the medium. Kjeldahl nitrogen (duplicates) was determined in aliquots of each preparation.

Assay of ATPase and apyrase activities. The activity of the mitochondrial ATPase was determined in a reaction medium containing in a total volume of 4 ml 25 mM succinate buffer, pH 6.5 or 7.5, 10 μmoles ATP, 10 μmoles $MgCl_2 \cdot 6\ H_2O$, 20 μmoles NaCl, 7 μmoles $CaCl_2 \cdot 2\ H_2O$, 20 mmoles sucrose and 0.5 ml mitochondrial suspension. The determinations were also performed in the same reaction medium but without sucrose and NaCl. In addition, in order to investigate whether this ATPase was Mg and/or Ca dependent, $MgCl_2$ or $CaCl_2$ were omitted on alternative occasions; *cf.* Baltscheffsky [16] and Saris.[17] The influence of added DNP was also studied. The reaction time was 15 min, and the incubation temperature +30°C. The reaction was stopped by the addition of 1 ml of ice-cold 20 % trichloroacetic acid. After centrifugation of the sample, an aliquot was taken for the determination of inorganic phosphate liberated, and the phosphate was estimated according to Teorell.[15]

The ATPase activities of the microsomes and of the soluble fraction were determined by using the same assay procedures. Apyrase activities were determined according to Krishnan [18] and to Cori *et al.*[11] The incubation temperatures used were +30° and +5°C. The latter temperature was used because Lee and Eiler [19] reported on a preparation from potatoes which, at temperatures above +7°, hydrolysed two acid labile phosphates in ATP and, at +7° or below, catalyzed the hydrolysis of only the terminal group.

RESULTS AND DISCUSSION

Rubin and Mikheeva [20] have observed a changed activity of cytochrome oxidase, polyphenol oxidase, and peroxidase in mitochondria isolated from γ-irradiated potato tubers. They assume that the high sensitivity of the

oxidative enzymes to gamma radiation is due to changes in the structure of the mitochondria.

Contradictory to this observation, Mukhin and Sal'kova [21] found that "oxidative processes proceeded at much the same rate in γ-irradiated and control potatoes". They concluded from special experiments that irradiation suppresses the primary phosphorylation of carbohydrates.

Kalacheva and Sissakian [22] found an influence of irradiation in mitochondria from pea seedlings only when the enzymes were tested under sub-optimal conditions.

Thus, the opinions differ on this matter. A necessary requirement therefore seemed to be that the mitochondria isolated from the control tubers were in a very active state and that optimal "working conditions" were established prior to an investigation of the influence of gamma radiation on the mitochondria. *Cf.* isolation procedure.

The oxygen uptake and the oxidative phosphorylation under optimal conditions. Mitochondria isolated from normal non-irradiated control tubers are called M_c and those isolated from γ-irradiated tubers M_i. As shown in Table 1,

Table 1. Determination of oxygen uptake and oxidative phosphorylation in mitochondria from control tubers (M_c) and tubers exposed to 14 kilorad γ-rays (M_i). Substrate: succinate. Each value represents a mean of 12 mitochondrial isolations performed in 1965.

Potato variety	Mito-chondria	Year of irradiation of tubers	pH of homo-genisation medium	mg N/ml mito-chondrial suspension	Uptake μatoms/h/mg N		P/O
					O	P	
King Edward	M_i	1963	7.85	1.44	33.9	59.2	1.75
»	M_i	1964	7.80	1.30	29.2	53.2	1.82
»	M_i	1965	7.90	1.32	26.9	53.3	1.98
»	M_c	—	7.90	1.20	34.1	62.0	1.82
Bintje	M_i	1964	7.80	0.72	50.6	98.3	1.97
»	M_i	1965	7.70	0.78	40.5	75.4	1.86
»	M_c	—	7.90	0.82	45.9	92.2	2.01
Early Puritan	M_i	1965	7.40	1.00	41.2	76.8	1.86
»	M_c	—	7.45	0.98	43.3	77.9	1.80

the oxygen uptake and the oxidative phosphorylation determined with succinate as substrate were equal for M_c and M_i. In both groups P/O ratios closely approached the theoretical value of 2. If malate alone was used as substrate, the oxygen uptake as well as the phosphorylation was exceedingly small. When, however, NAD^+ or NADH was added together with malate, the system functioned quite normally and P/O ratios up to 3 were obtained (Table 2). As mentioned before, nitrogen determinations were performed for each single preparation. The values for oxygen and phosphorus uptake in Tables 1 and 2 are given per mg N in the mitochondrial suspension.

Isolation of mitochondria, oxygen uptake, and oxidative phosphorylation carried out under sub-optimal conditions. P/O ratios closely approaching the

Table 2. Determination of oxygen uptake and oxidative phosphorylation in mitochondria isolated from control tubers (M_c) and tubers exposed to 14 kilorad γ-rays (M_i). Substrate: malate + NAD^+ or malate + NADH. Mean of 10 isolations.

Potato variety	Mitochondria	pH of homogenisation medium	mg N/ml mitochondrial suspension	Uptake μatoms/h/mg N O	Uptake μatoms/h/mg N P	P/O
Bintje	M_i	7.55	0.90	37.3	110.2	2.95
»	M_c	7.90	1.16	25.5	76.8	3.00
King Edward	M_i	7.90	0.62	17.4	50.3	2.89
»	M_c	7.80	0.85	18.7	54.9	2.93
Early Puritan	M_i	7.70	0.82	43.4	128.2	2.96
»	M_c	7.90	0.98	30.6	91.0	2.97

theoretical value of 2 with succinate and of 3 with malate + added NAD as substrate, could be obtained only with mitochondria isolated in the pH range 7.2—7.9 and with a pH of 7.2—7.5 in the reaction medium. At pH 6.5, a level, by Verleur [9] reported to give mitochondria in the best condition, only a normal oxygen uptake was observed in the present investigation, the phosphorylative capacity, however, being extremely low. At a pH higher than 8, the oxygen uptake, as well as the phosphorylation was depressed. Table 3 illustrates the P/O ratios at different pH's with succinate as substrate. Similar results were obtained when malate + NAD were used. As can be seen from Table 3, the activities of enzymes in M_i are somewhat more affected by the low pH than those in M_c. This result is in accordance with the reports of Kalacheva and Sissakian [22] mentioned above.

Table 3. Determination of oxygen uptake and oxidative phosphorylation in mitochondria isolated from control tubers (M_c) and tubers exposed to 14 kilorad γ-rays (M_i) at different pH of the homogenisation medium by isolating the mitochondria. Substrate: succinate. Single isolations.

Potato variety	Mitochondria	pH of homogenisation medium	mg N/ml mitochondrial suspension	Uptake μatoms/h/mg N O	Uptake μatoms/h/mg N P	P/O
King Edward	M_i	7.10	0.90	43.5	53.0	1.22
»	M_c	7.00	0.80	33.0	43.9	1.32
»	M_c	7.95	1.12	35.7	71.4	2.00
Bintje	M_i	7.15	1.02	36.8	37.4	1.02
»	M_c	7.15	1.24	37.3	45.9	1.23
»	M_c	7.90	0.78	45.6	90.8	1.99
»	M_i	7.85	0.86	40.9	85.1	2.08
Early Puritan	M_i	7.10	1.01	43.3	56.7	1.31
»	M_c	7.30	1.18	40.3	70.8	1.76
»	M_c	8.00	0.76	44.2	87.4	1.98
»	M_i	7.50	1.01	44.4	84.5	2.02

Table 4. Determination of oxygen uptake and oxidative phosphorylation in mitochondria isolated from control tubers (M_c) and tubers exposed to 14 kilorad γ-rays (M_i). 10^{-4} M DNP present in the reaction medium. Substrate: succinate. Mean of 8 isolations.

Potato variety	Mitochondria	pH of homogenisation medium	mg N/ml mitochondrial suspension	Uptake μatoms/h/mg N O	Uptake μatoms/h/mg N P	P/O
Bintje	M_i	7.35	1.02	35.7	25.5	0.71
»	M_c	7.35	1.06	37.7	41.9	1.11
Early Puritan	M_i	7.50	1.01	33.6	39.1	1.16
»	M_c	7.45	1.18	43.3	56.7	1.31
King Edward	M_i	7.35	1.10	37.3	45.9	1.23
»	M_c	7.55	1.15	33.0	43.9	1.32

Effect of the uncoupling agent DNP. The uncoupler DNP depressed the P/O ratios to greater extent when M_i were used than when M_c were used. As expected, the oxygen uptake was quite normal in both groups; the phosphorylation, however, was somewhat more inhibited in the case of the M_i. Table 4 gives the results of the measurements of the oxidation and phosphorylation rates, in the presence of DNP, of mitochondria prepared from control tubers and from γ-irradiated tubers. In this case there is a significant difference in P/O ratios between M_i and M_c.

Most investigators seem to agree that the true uncoupling agents act by causing a breakdown of some high-energy intermediate. The reason for the different influence of DNP on M_i and M_c might perhaps be that a disturbed metabolism of the extremely sensitive high-energy intermediate is more pronounced in the irradiated tuber tissue. It has also been shown that the influence of M_i on amino acids in press-juices of potatoes differs from that of M_c (unpublished results). It might be possible that some amino acid transaminating enzyme associated with the Krebs cycle, functions as protector of the high-energy intermediate. Thus, a change in this respect caused by the irradiation could explain the different effect of DNP on M_i and on M_c.

Activities of ATPase and apyrase. The ATPase activity in the mitochondrial fraction was negligible, a criterion of the isolated mitochondria being in an intact state. The ATPase activity of the microsomal fraction was higher. Additions of DNP had no effect on the activity. Moreover the activity seemed to be independent of Ca and Mg. The amount of inorganic phosphate liberated was, however, higher than the amount corresponding to the terminal phosphate of ATP which should have been released by a true ATPase. It was even higher than the acid labile phosphate determined in the reaction mixture (7 min hydrolysis in 1 N HCl at 100°C), indicating the presence of a phosphatase or some other phosphate hydrolysing enzyme. In fact, by using AMP as substrate, inorganic phosphate was shown to be liberated also at pH 7.5.

Further, apyrase preparations isolated as described above were not obtained in an enzymatically homogeneous state. The best results were achieved by using the method of Cori *et al.*,[11] especially Procedure II described by these

authors. Apyrase obtained from tubers of the King Edward variety (irradiated with the dose 14 kilorad) had a somewhat increased activity as compared to apyrase isolated from non-irradiated tubers of the same variety. The amount of inorganic phosphate released at +5°C was half the amount released at +30°C, which is in accordance with reports by Lee and Eiler [19] mentioned above. The number of enzyme isolations is, however, too small to give statistical significance. Additional experiments are required before decisive conclusions can be drawn. Further studies on this question are in progress.

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