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Preparation and Some Properties of Sub-Mitochondrial, Reduced Diphosphopyridine Nucleotide Oxidizing Particles from Isolated Mitochondria

HERRICK BALTSCHEFFSKY, MOLLY FUDGE and BARBRO ARWIDSSON

Wenner-Gren Institute, University of Stockholm, Sweden

A simple method for preparation and isolation of sub-mitochondrial particles from isolated rat or guinea-pig liver mitochondria is described. It consists of grinding the mitochondria with Al_2O_3 in a mortar and subsequent differential centrifugation. Some properties of a fraction, which is centrifuged down at 100 000 *g* (60 min centrifugation), are described.

The fraction oxidizes reduced diphosphopyridine nucleotide at a much higher rate than reduced triphosphopyridine nucleotide, both with oxygen and with cytochrome c as electron acceptors. The oxidation of succinate with cytochrome c is strongly stimulated by phenazine methosulfate. Antimycin A inhibits the oxidation of succinate in the absence of phenazine methosulfate, which can mediate electron transport from succinate to cytochrome c around the antimycin A-sensitive site. When a catalytic amount of diphosphopyridine nucleotide is added, β-hydroxybutyrate is oxidized by added cytochrome c in the presence of the sub-mitochondrial fraction whereas glutamate oxidation occurs only when also the final supernatant is added. This indicates that mitochondrial dehydrogenases are differently distributed in the sub-mitochondrial fractions.

The methods employed for preparation of sub-mitochondrial particles from isolated animal mitochondria [1–10] are of two kinds with respect to the disruption of the mitochondrial structure, chemical [1–6] and physical or mechanical [7–10]. Three physical methods have been reported: use of a Waring blendor[7], sonic vibration [8–9] and grinding with alumina [10]. This paper describes the method of preparation and some properties of sub-mitochondrial DPNH-oxidizing * particles obtained from isolated liver mito-

* Abbreviations: ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DNP, 2,4-dinitrophenol; PMS, phenazine methosulfate; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; M, moles per liter.

chondria by grinding with alumina. Earlier communications on this method have been in note or abstract form [10-13].

MATERIALS AND METHODS

The pyridine nucleotides, PMS, cytochrome c and antimycin A were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. HOQNO was obtained from Dr. J. W. Cornforth, National Institute for Medical Research, London, England, this gift is gratefully acknowledged. Antimycin A and HOQNO were dissolved in ethanol. The stock solutions were kept in a deep-freeze. The molecular weight of antimycin A was taken as 548, in agreement with the formula $C_{28}H_{40}O_9N_2$ [14] (*cf.* Ref.[15]).

Liver mitochondria were prepared in 0.25 M sucrose, essentially according to the method of Schneider and Hogeboom [16] as modified by Ernster and Löw [17]. For one preparation 4—8 white male rats, each weighing 200—300 g or 2—4 male guinea-pigs, each weighing 300—400 g were used. After decapitation of an animal, the liver was removed, weighed, cut into small pieces and rinsed once or twice with sucrose. The homogenization was carried out on a 30—40 % tissue suspension as rapidly as possible (in between 15 and 30 sec) in a Potter-Elvehjem homogenizer with a loosely fitting Teflon Pestle. When all the livers were homogenized a dilution to a 15—20 % suspension was made. The material was centrifuged 10 min at 800 *g* * in an International Refrigerated Centrifuge, model PR-1, head no. 840. The pellet was discarded and the supernatant centrifuged 20 min at 4 100 *g*. The mitochondrial pellet was suspended in sucrose to the same volume as before the centrifugation and recentrifuged at 4 100 *g*, now for 15 min. The supernatant was discarded and the mitochondrial pellet suspended in a small volume of sucrose (1—2 ml per 5 g of liver). The material was centrifuged 8 min at 20 000 *g* in a Spinco Ultracentrifuge, model L, head no. 40, which was also used for the subsequent centrifugations. In this centrifugation the resulting pellet was sharply divided into two parts. The upper part, small in volume, was the remaining "fluffy layer" [18] which was thoroughly swirled off from the densely packed mitochondrial layer and discarded. The remaining layer contained mitochondria of high ("intact") quality, as judged by the criteria of P/O ratios and respiratory control. It was used for preparation of the sub-mitochondrial particles, according to one of the following two methods.

Method I: The mitochondrial layer was suspended in a small volume of 0.03 M phosphate buffer, pH 7.0, and centrifuged 5 min at 25 000 *g*. The supernatant was discarded. The pellet was mixed in a mortar with 20 mg Al_2O_3 (Alcoa A-301) per gram liver and ground for 6 min. The mixture was transferred to a centrifugation tube with small volumes of the phosphate buffer and centrifuged 10 min at 25 000 *g*. The pellet contained Al_2O_3 and "heavier fragments". The supernatant was centrifuged 60 min at 100 000 *g*. The pellet from this centrifugation was suspended in a small volume of the phosphate buffer. The resulting yellow, translucent suspension contained "DPNH-oxidase particles".

Method II: The mitochondrial layer was mixed in a mortar with 20 mg Al_2O_3 (Alcoa A-301) per gram liver and ground for 6 min. The mixture was transferred to a centrifugation tube with small volumes of 0.25 M sucrose. From this on, the method was the same as method I, except that 0.25 M sucrose was used throughout.

The "heavier fragments" could be separated from the alumina with a glass rod. This fraction was obtained by transfer to an empty tube with the aid of phosphate or sucrose medium, 10 min centrifugation at 25 000 *g* and suspension of the pellet in the medium desired.

All operations were carried out at as near to 0°C as possible. Delays between the individual steps were avoided.

The solutions were made with deionized water and kept at 0°C.

Method II has also been used for preparation of sub-mitochondrial particles from isolated rat- or guinea pig-heart-muscle mitochondria [12].

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* The *g* values are given for the bottom of the tubes with the International Refrigerated Centrifuge and for the middle of the tubes with the Spinco Ultracentrifuge.

Electron micrographs were taken of the isolated mitochondria, the "heavier fragments" fraction and the "DPNH-oxidase particles" *. The majority of the mitochondria appeared to be "intact" with clearly visible internal double membrane structures. Some, however, appeared to be swollen, with diminished integrity of the internal membranes. The microsomal contamination appeared to be low. Both the "heavier fragments" fraction and the DPNH-oxidase particles appeared to be more or less round vesicles with no interior structures. No "intact" mitochondria were observed in these fractions.

Measurements. Protein was determined by the biuret method. Phosphorylation was measured by the ^{32}P method recommended by Lindberg and Ernster [19], ATP-ase activity by the method of Martin and Doty [20] as described by Lindberg and Ernster [19].

Oxidase activities were measured with the Warburg technique or, for DPNH and TPNH by following the disappearance of the absorption of reduced nucleotide at 340 mμ in a Beckman Recording Spectrophotometer, model DK 2. Cytochrome c reducing activities were measured at 550 mμ, in the presence of mM KCN. The following values were used for the extinction coefficients: DPNH minus DPN and TPNH minus TPN at 340 mμ = 6.2 $cm^{-1} \times mM^{-1}$, reduced cytochrome c minus oxidized cytochrome c at 550 mμ = 19.1 $cm^{-1} \times mM^{-1}$.

RESULTS

A method similar to method I where the mitochondria were treated with 0.03 M phosphate, pH 7.0, before the grinding, was outlined in the first note [10] about this work. This medium had earlier been used for preparation of submitochondrial particles by sonic treatment of liver mitochondria [21]. Method II gave a higher yield than method I (Table 1), as was previously noted [11]. Preparations made according to method II have been used for the experiments reported below, unless otherwise indicated.

Table 1. Yield of "DPNH-oxidase particles".

Material	Method	Number of preparations	mg protein/g liver		
			lowest value	highest value	mean value
Rat liver	I	3	0.1	0.2	0.1
» »	II	9	0.5	0.9	0.7
Guinea pig liver	I	3	0.2	0.3	0.2
» » »	II	4	0.8	1.4	1.1

The higher yield obtained with method II allowed some experiments to be done in order to confirm the visual observation that 6 min was a suitable grinding time. Mitochondria were isolated from 80 g of liver. During the grinding, which was extended to 15 min, samples were removed from the mixture at suitable time intervals, weighed and put into centrifuge tubes. Two variables of the "DPNH-oxidase particles" obtained from these samples were determined: the amount of protein recovered and the DPNH-oxidase activity. Fig. 1 shows that 6 min grinding was suitable with respect to the yield of

* Thanks are due to Dr. Fritiof S. Sjöstrand of the Department of Anatomy, Karolinska Institutet, for carrying out the studies with the electron microscope.

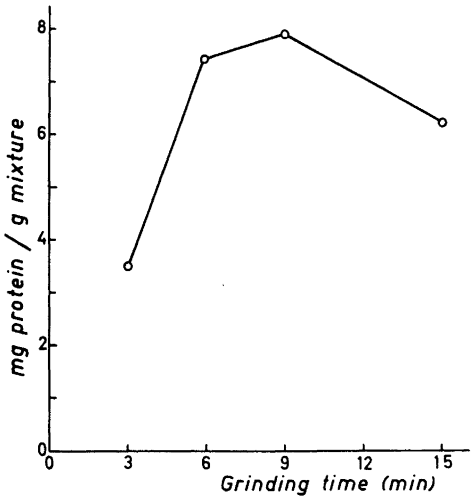

Fig. 1. Yield of protein as a function of grinding time (guinea pig).

protein. Table 2 shows that this was true also with respect to the yield of DPNH-oxidase activity. Parallel values are given in Table 2 to show that the experimental error was satisfactorily small for the purpose of the experiment.

The recovery of mitochondrial protein is shown in Table 3. With both methods of preparation the bulk of the protein was in the supernatant after the centrifugation at 100 000 *g*. Whereas method II gave a better yield of "DPNH-oxidase particles" than method I, the opposite was true for the "heavier fragments" fraction. A considerable amount of protein (6 %) was dissolved by the treatment of the "intact" mitochondria with phosphate. As was to be expected, the amount of protein was low (2 %) in the corresponding fraction from a similar centrifugation in 0.25 M sucrose.

The efficiency of the oxidative phosphorylation obtained was very low, as was shown in a note [10]. It was true both with preparations made according to method I and method II, and likewise when versene was added or a mixture of sucrose and phosphate was used. Shortening the reaction time to

Table 2. DPNH-oxidase activity as a function of grinding time. The oxidation of DPNH was measured spectrophotometrically at 340 mμ, in 25 mM phosphate buffer pH 7.0, in the presence of 1 % serum albumin. Material: "DPNH-oxidase particles". Each given value is the mean value of two determinations from a separate sample. Reaction volume 3 ml (guinea pig).

Grinding time min	DPNH-oxidase activity μmoles DPNH oxidized/min/g mixture	
0.2	0.1	0.3
3	1.7	1.6
6	2.0	2.5
9	2.5	2.6
15	2.3	2.2

Table 3. Recovery of mitochondrial protein (rat).

Fraction	Recovery per 100 mg mitochondrial protein	
	Method I	Method II
	mg protein	mg protein
"DPNH-oxidase particles"	7	23
"Heavier fragments"	15	11
Supernatant$_{25\,000\ g}$ *	6	2
Supernatant$_{100\,000\ g}$ *	59	55
Total recovery	87	91

* Supernatant $_{25\,000\ g}$ and Supernatant $_{100\,000\ g}$ = supernatant from the centrifugation at 25 000 *g* which was performed immediately before the grinding and the centrifugation at 100 000 *g*, respectively.

2 min (measuring the rate of oxidation of DPNH at 340 mμ and at 20°C), did not result in higher P/O ratios.

The ATP-ase activity of isolated liver mitochondria is related to their state and capacity for oxidative phosphorylation. A parallel relationship exists between disappearance of mitochondrial integrity, oxidative phosphorylation and DNP-induced ATP-ase activity. When these decrease, the Mg^{++}-stimulated ATP-ase activity, on the other hand, increases [22]. Fig. 2 shows that the Mg^{++}-stimulated ATP-ase activity of the "DPNH-oxidase particles" is very high as compared to the DNP-induced ATP-ase and the endogenous ATP-ase activities.

In early preparations of "DPNH-oxidase particles" with the alumina grinding-method, DPNH was oxidized at a much higher rate than succinate with either oxygen or cytochrome c as electron acceptor [10,11]. It has been

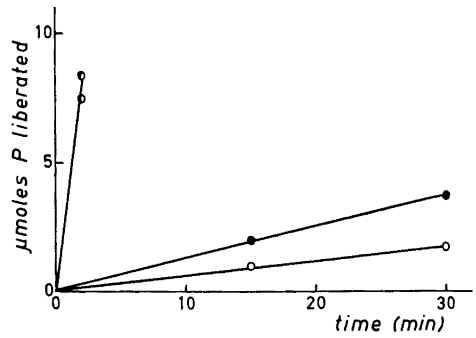

Fig. 2. Effects of Mg and DNP on the ATP-ase activity. ◐ = Mg added, ● = DNP added, O = no addition. The reaction medium contained: 0.015 M tris(hydroxymethyl)-aminomethane, pH 7.6, and 0.01 M ATP. Where added: 4 mM $MgCl_2$ or 10^{-4} M DNP. Final volume 2.0 ml. Temperature 30°C (guinea pig).

Table 4. Cytochrome c reductase activities. Spectrophotometric measurement at 550 mμ. In the reaction cuvette: "DPNH-oxidase particles", 1 mM KCN, 5×10^{-5} M cytochrome c and 30 mM phosphate buffer, pH 7.0. Where added: 6.7 mM succinate or 0.1 mM DPNH (final concentrations are given throughout). The final volume was 3 ml. Temperature 22°C. The values are given as μM cytochrome c reduced/min/mg protein (rat).

Method	Number of preparations	DPNH-cytochrome c reductase activity			Succinate-cytochrome c reductase activity			
		lowest value	highest value	mean value a	lowest value	highest value	mean value b	$\frac{a}{b}$
I	3	120	260	200	4.5	12	9.2	22
II	6	140	390	220	4.2	19	10	22

Table 5. Cytochrome c-reducing activities in "DPNH-oxidase particles". The reduction of cytochrome c was measured spectrophotometrically at 550 mμ. In the cuvette: "DPNH-oxidase particles", 1 mM KCN, 5×10^{-5} M cytochrome c and 30 mM phosphate buffer, pH 7.0, and, where added: 6.7 mM succinate, 6.7 mM glutamate, 6.7 mM β-hydroxybutyrate, 0.1 mM DPNH, 0.1 mM TPNH, 0.033 mM DPN (with glutamate and β-hydroxybutyrate), 0.01 M DPN (with TPNH), 0.33 mM PMS, 0.67 μM antimycin A, 0.2 ml supernatant (sup) containing 5.5 mg protein per ml. Temperature 22°C. The additions were made during the experiment (rat).

Substrate	Addition	Addition	μM cytochrome c reduced/min/mg protein
Succinate	—	—	5
Sup	—	—	13
Succinate	Sup	—	17
Succinate	—	—	5
Succinate	PMS **	—	45
Succinate	Antimycin A	—	0
Succinate	Antimycin A	PMS	51
DPNH			130
Glutamate	—	—	0
Glutamate	DPN	—	0
Glutamate	DPN	Sup	79
β-Hydroxybutyrate	—	—	0
β-Hydroxybutyrate	DPN	—	47
β-Hydroxybutyrate	DPN	Sup	54
TPNH *	—	—	6
TPNH *	DPN	—	30
DPNH *	—	—	115

* Experiments made on preparations according to method I.

** As PMS is autoxidizable the data with PMS may be quantitative only with respect to the reduction of cytochrome c (and not indicate an equivalent oxidation of succinate).

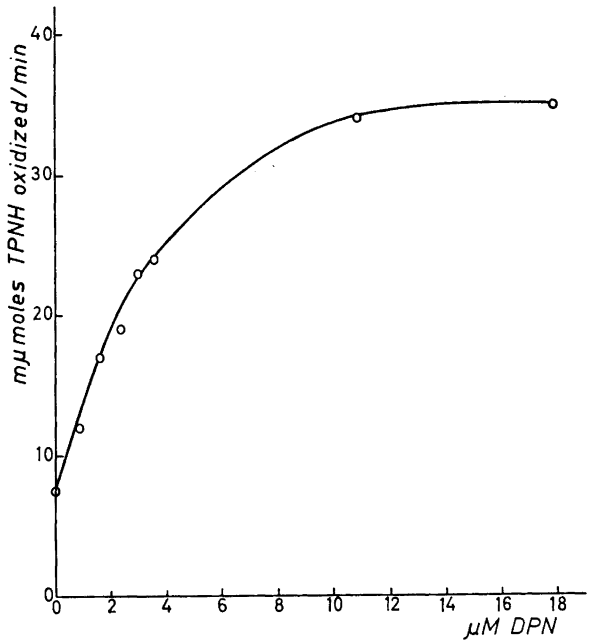

Fig. 3. Stimulation of TPNH-oxidase by DPN. The oxidation of TPNH was measured spectrophotometrically at 340 mμ. Initial concentration of phosphate buffer pH 7.0, was 27 mM. Protein content 1.9 mg. Successive additions of DPN were made. Final volume before the additions 3.0 ml. Note the rate of oxidation of TPNH (that of DPNH was, in a separate experiment, 120 mμmoles DPNH oxidized/min). (Variation of the concentration of phosphate buffer over a wide range did not influence the rate.) Temperature 20°C (rat).

found, however, that both method I and method II may give preparations where the oxidase activities were of the same order of magnitude with succinate and with DPNH as substrates. On the other hand, the difference between the rates with DPNH and succinate as substrates has constantly been great for the cytochrome c reductase activities (Table 4).

Different cytochrome c-reducing activities are shown in Table 5. The low rate of oxidation of succinate with cytochrome c is stimulated by phenazine methosulfate, which is known to accept electrons from a purified and solubilized succinic dehydrogenase preparation [23]. Antimycin A (or HOQNO) inhibits the succinate-cytochrome c reductase activity, but the stimulation due to phenazine methosulfate is unihibited. These agents have previously been reported to provide a by-pass for electrons around the antimycin A- and HOQNO-sensitive step in bacterial light-induced phosphorylation [24,25], and recently also in mitochondrial electron transport [26]. The present results demonstrate that electrons which, through the action of phenazine methosulfate, have passed around the antimycin A sensitive step may be brought back to the respiratory chain at the cytochrome c-level. Mitochondrial substrates,

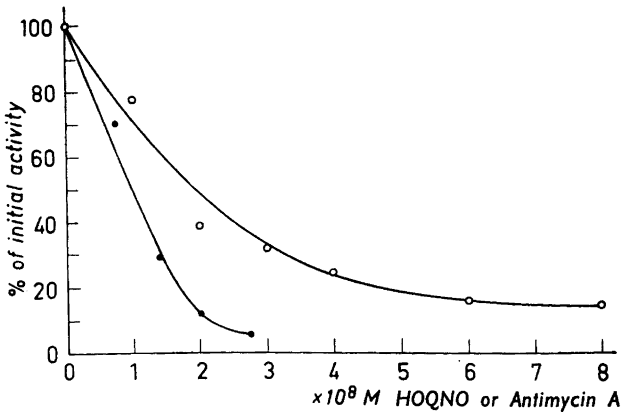

Fig. 4. Inhibition by antimycin A and HOQNO of DPNH-oxidase activity. ○ = HOQNO, ● = antimycin A. The oxidation of DPNH was measured spectrophotometrically at 340 mμ. The reaction medium contained: 25 mM phosphate buffer pH 7.0 and "DPNH-oxidase particles" containing 0.62 mg protein. Final volume 3.0 ml. Temperature 22°C (rat).

which are linked to DPN over specific dehydrogenases, may be oxidized after addition of catalytic amounts of DPN (*e.g.* β-hydroxybutyrate), or they may need addition of both DPN and the final supernatant from the preparation (*e.g.* glutamate). Mitochondrial dehydrogenases thus seem to be distributed in different sub-mitochondrial fractions. This may be a reflection of a corresponding compartmentation in the structure of the mitochondrion. The low initial rate of oxidation of TPNH can be markedly stimulated by DPN in catalytic amounts. This is shown in more detail in Fig. 3 and indicates that pyridine nucleotide transhydrogenase [27] is present in the particles.

It has been shown earlier [12] that amytal *, antimycin A, and HOQNO in "DPNH-oxidase particles" from liver inhibit only that part of the respiration which is not due to the addition of cytochrome c. Fig. 4 shows the inhibition curves for antimycin A and HOQNO in a system where no cytochrome c had been added.

DISCUSSION

Isolated "intact" mitochondria have been disrupted into smaller fragments by various chemical and mechanical means. The new, simple method described in this paper, grinding with alumina, was originally used for disruption of bacterial cells [28]. After the grinding the various sub-mitochondrial fractions are separated by differential centrifugation performed in a similar manner to that after sonic disruption [8]. The recovery of mitochondrial protein in different fractions (Table 2), with a high amount in the final supernatant, resembles the distribution shown by Kielley and Bronk [8] in the fractions obtained from sonicated mitochondria.

* 5-Ethyl-5-*iso*amylbarbiturate.

The fraction which has been termed "DPNH-oxidase particles" oxidized DPNH in previously described preparations at a much higher rate than succinate [10]. As many preparations, both with method I and method II, have given rather similar rates with the two substrates and as a marked variation between different preparations has been found, it can be stated that the alumina grinding as such does not constantly give low succinoxidase activity in the "DPNH-oxidase particles" and the absolute DPNH-oxidizing activities are governed by factors which are difficult to control. Two possible sources for this difficulty should be considered, the postulated "external" mitochondrial pathway for oxidation of DPNH [29,30], and influence of microsomes. The fact that amytal, antimycin A and HOQNO virtually completely inhibit the DPNH-oxidase activity in the absence of cytochrome c [11,12] shows that this respiration is of a mitochondrial, and not microsomal, origin. The additional respiration, which is initiated by external cytochrome c, may originate from either of the two sources mentioned above, or from both. A low microsomal contamination in the mitochondria would be percentagewise increased in the fraction containing the "DPNH-oxidase particles" because of similar sedimentation characteristics. DPNH-cytochrome c reductase activity of the microsomes may then be linked to the mitochondrial oxidase at the cytochrome c-level and by-pass a rate-limiting step between DPNH and cytochrome c. The effect would be the same as that of the postulated "external" mitochondrial pathway. A vague indication that microsomes may be responsible for at least part of the stimulating effect of added cytochrome c in preparations from liver is found from experiments with "DPNH-oxidase particles" from heart muscle [12]. In this material the increased DPNH-oxidase activity, which is due to addition of cytochrome c, is sensitive to the above-mentioned inhibitors, and the ratio DPNH- to succinate-cytochrome c reductase activity is lower than the corresponding ratio in liver material. The amount of microsomes which can be obtained from heart muscle homogenates is very low.

Glutamate was not oxidized by the DPNH-oxidase particles unless both DPN and the final supernatant were added. Thus no glutamic dehydrogenase was active in the particles. The same was reported for sub-mitochondrial particles obtained by Kielley and Bronk [8] (but not by McMurray, Maley and Lardy [9]) after sonic disruption of liver mitochondria. On the other hand, β-hydroxy-butyric dehydrogenase was present (Table 5).

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