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Abstract

1. After the centrifugation of sonicated heavy beef heart mitochondria at 75, 000 × g for 10 minutes, the supernatant was centrifuged at 144, 000 × g for 30 minutes. The residue was revealed being composed of vesicular inner membrane fragments (ETPH), about 600 to 1000 Å. in diameter, showing a morphological homogeneity and a high capacity of oxidative phosphorylation. 2. The Pia ratio of the ETPH in the presence of succinate and of NADH2 was 1.68 and 2.54, respectively, and the corrected Pia value for O2 gas equilibrium was 1. 01 and 1.40, respectively. 3. The capacity of oxidative phosphorylation in ETPH fraction was parallel to the activity of the oligomycin. sensitive ATPase in these fractions. 4. The P/0 ratio of ETPH was decreased to about 50 % by hypotonic treatment. The decrease of P/0 ratio was restored to the level of about 90 % by incubating the ETPH with ATP and BSA. In the instance where the P/0 ratio was low level in the hypotonic medium, the surface structure of ETPH was observed as a swollen form and the head pieces of the elementary particles were clearly observed in contrast to the solid surface structure of ETPH in the isotonic medium. 5. The P/0 ratio of ETPH was decreased to about 60 % by relatively severe sonication, and after separating the residue from the supernatant, that of the residue decreased further to about 40 %. The P/0 ratio of the residue was restored to the level before the separation on the addition of the supernatant containing oligomycin-insensitive ATPase. 6. A discussion was made on the correlation between the surface structure and the activities at terminal phosphorylation step of ETPH after the simple physico-chemical treatment.

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CORRELATION OF STRUCTURE AND FUNCTION IN THE OXIDATIVE PHOSPHORYLATION SYSTEM OF SUBMITOCHONDRIAL PARTICLES

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Since the isolation of the submitochondrial particles capable of oxidative phosphorylation by Green *et al.* (1) and Lehninger *et al.* (2) in 1956, the preparation method (3—5) of the submitochondrial particles has been established. Up to the present, it has been demonstrated that the submitochondrial particles are the minimal structural units which equip the function of respiratory chain phosphorylation, and hence the study of the correlation between the structure and function of the submitochondrial particles would be important for the elucidation of the mechanism of the respiratory chain phosphorylation.

Two hypotheses are offered for the explanation of the mechanism of the respiratory chain phosphorylation. The one is a chemical hypothesis (flow sheet type) (6, 7) and the other, a chemiosmotic hypothesis recently proposed by Mitchell (8—10). One of the fundamental differences in these hypotheses lies in the explanation for the primary high energy state in the respiratory chain phosphorylation. Namely, the former explains the primary high energy state as a chemical intermediate while the latter as an electric membrane potential. However, both hypotheses take commonly into account the participation of ATPase in the terminal step of the respiratory chain phosphorylation. It has recently been confirmed (11—15) that the head pieces of the elementary particles of the mitochondrial inner membrane (16, 17) is a substance identical with ATPase, a coupling factor F_1 (18). These papers imply that the minimal structural units, the head pieces attached on the inner membrane, are essentially required in the respiratory chain phosphorylation. It is very interesting, therefore, to study the correlation of the change of the membrane structure and that of the capacity of the respiratory chain phosphorylation in the submitochondrial particles.

This paper describes the results of the reexamination done on the

isolation of the submitochondrial particles which show the high capacity of respiratory chain phosphorylation and morphological homogeneity. In addition, some comments are made on the correlation between the capacity of respiratory chain phosphorylation and the activity of oligomycin-sensitive ATPase in the submitochondrial particles. The relation between the change of the membrane structure and that of the capacity of respiratory chain phosphorylation after simple physico-chemical treatments on the submitochondrial particles have also been investigated.

MATERIALS AND METHODS

Isolation of mitochondria

Heavy mitochondria (HBHM) were isolated from beef heart by the modification of the method of CRANE *et al.* (19)

Preparation of submitochondrial particles (ETPH)

The suspension of HBHM (20 mg protein/ml of 10 mM Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose, 1 mM succinate, 1 mM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$) (20) was sonicated for 15 seconds/ml/20 mg protein at maximum intensity by an ultrasonicator (Kaijo Electric Co., LTD, 20 KC, 150 W, 7 ϕ tip). The sonicated mitochondrial suspension was centrifuged at varying gravity, and the physico-chemical properties of the residue (ETPH) and the supernatant were observed.

Determination of the capacity of respiratory chain phosphorylation

The capacity of the respiratory chain phosphorylation of the submitochondrial particles was determined in a glucose trapping system with a Warburg manometer (21). The reaction mixtures were composed of 750 μmoles sucrose, 25 μmoles succinate (or 100 μmoles ethanol, 2 μmoles NAD and 0.5 mg alcohol dehydrogenase), 20 μmoles potassium phosphate buffer (pH 7.5), 5 μmoles $MgCl_2$ and the submitochondrial particles in the main chamber, and 5 μmoles ADP (or ATP), 100 μmoles glucose and 200 K. M. units hexokinase in the side arm 1. The total volume of the reaction mixture was 3.0 ml. The incubation mixture was preincubated at 25° for 8 minutes, and then the reaction was started by adding ADP, glucose and hexokinase. The oxygen uptake was estimated every 4 minutes and after 8 minutes the reaction was stopped by transferring 0.3 ml of 5.6 M trichloroacetic acid from the side arm 2 to the main chamber, and the oxygen consumption was estimated further for 12 minutes for the correction of O_2 gas equilibrium.

Assay of ATPase

The reaction mixture was composed of 50 mM Tris-HCl buffer (pH 7.5), 6 μmoles ATP, 3 μmoles $MgCl_2$ and 400 μg protein of ETPH and total volume was 1.0 ml, and incubated at 30° for 10 minutes.

Chemical analysis

Inorganic orthophosphate was determined by the method of MARTIN and

DOTY (22) modified by TAKAHASHI (23) and protein was estimated by the method of LOWRY *et al.* (24)

Electron microscopy

Usually, after negative staining of the submitochondrial particles with 1 % potassium phosphotungstate (25, 26), the specimen was observed by a Hitachi 11 C type electron microscope.

Chemicals

Hexokinase, ATP, ADP, bovine serum albumin (BSA) and phosphotungstic acid were obtained from Boehringer, Sigma, Armour and Merck. Oligomycin was kindly provided by Dr. D. E. Green. All other chemicals used were of analytical grade.

RESULTS

Physico-chemical properties of isolated submitochondrial particles

The centrifugation of the sonicated heavy beef heart mitochondria was conducted for 10 minutes at 16, 000×g and followed by the centrifugation of the supernatant (Sap 16) for 60 minutes at 198,000×g and the residue thus obtained, the submitochondrial fraction (ETPH), occupied about 30 % of the total protein of HBHM. However, this ETPH proved to be heterogenous in size and form, and its capacity of the oxidative phosphorylation to be rather low. Therefore, it is necessary to prepare ETPH fraction which is in somewhat homogeneous size and form and has higher capacity of oxidative phosphorylation. About 34 % of the total submitochondrial fraction was recovered in the residue (R_{75-105}) centrifuged at 105, 000×g for 30 minutes from the supernatant following the centrifugation of the sonicated HBHM at 75, 000×g for 10 minutes (Fig. 1).

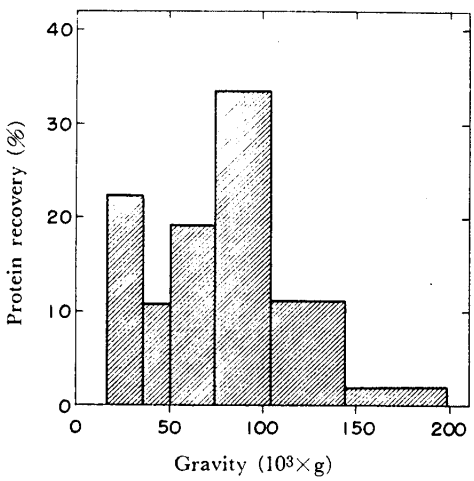


Fig. 1 Distribution of the submitochondrial particles prepared by differential centrifugation of supernatant subsequent to the centrifugation of sonicated heavy beef heart mitochondria at 16,000×g for 10 min. Time of centrifugation; 10 min at the gravity less than 75,000×g, 30 min at 105,000×g and 144,000×g and 60 min at 198,000×g

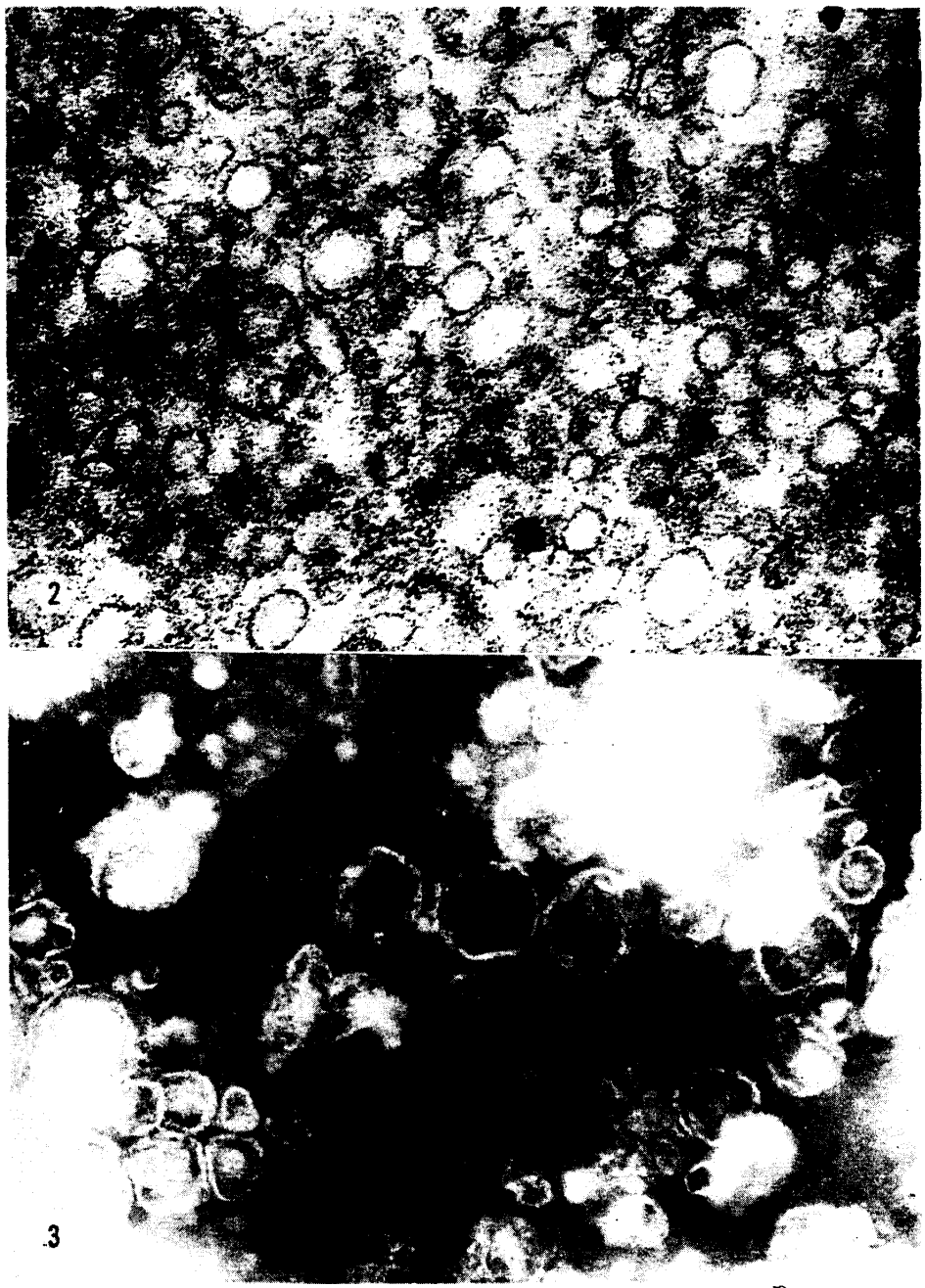


Fig. 2 Electron micrograph of the submitochondrial (R_{75-144}) fraction. The specimen was fixed with osmium tetroxide, embedded in Epon, sectioned, and stained with uranyl acetate. Ferritin was mixed just before the fixation. Magnification ×66,000

Fig. 3 Electron micrograph of the submitochondrial (R_{75-144}) fraction. The (R_{75-144}) fraction was fixed with osmium tetroxide (final 1.6%) for 50 min, washed with distilled water and centrifuged at 144,000×g for 15 min, and the residue was negatively stained with 1.5% PTA. Vesicular membraneous structure is observed. Magnification ×121,000

The highest capacity of the oxidative phosphorylation was observed in the residue fraction ($R_{105-198}$) by the centrifugation (at 198, 000×g for 60 minutes) of the supernatant subsequent to the centrifugation of the sonicated HBHM at 105, 000×g for 30 minutes (Table 1).

Table 1 Succinoxidase activity and P/O ratio in submitochondrial fraction obtained from sonicated heavy beef heart mitochondria

Submitochondrial fractions were prepared from HBHM stocked in −20° for 1 to 20 days.

Expt. No.	Fraction*	Protein recovery (%)	Succinoxidase activity (mμmoles/min/mg protein)	P/O**
1	R_{16-36}	22.5	187	0.17
	R_{36-105}	64.5	148	0.60
	$R_{105-198}$	13.1	157	1.15
2	R_{36-105}	64.5	206	0.80
	$R_{105-144}$	11.3	266	0.88
	$R_{144-198}$	1.9	120	0.23
3	R_{50-75}	19.3	131	0.56
	R_{75-144}	45.0	162	1.01
	R_{75-198}	46.9	167	1.12

* R_{x-y} is abbreviation of the residue fraction obtained by the centrifugation ($y \cdot 10^3 \times g$) of the supernatant subsequent to the centrifugation of sonicated HBHM at $x \cdot 10^3 \times g$. For details of centrifugation, see Fig. 1.

** With succinate. The correction for O_2 gas equilibrium was usually conducted as described in METHODS.

After the centrifugation of the sonicated HBHM at 75, 000×g for 10 minutes, the supernatant was centrifuged at 144, 000×g for 30 minutes and the residue fraction (R_{75-144}) served as standard ETPH, because it was relatively homegeneous in size and form, measuring 600 to 1000 Å in diameter as shown in Figs. 2 and 3, and had relatively high capacity of the oxidative phosphorylation. The recovery rate of this ETPH was about 15 % of the original HBHM.

The difference spectrum of the ETPH and the content of cytochromes per mg protein of the ETPH are shown in Fig. 4 and Table 2.

Table 3 shows the P/O ratio of the ETPH in the presence of succinate and in the generating system of $NADH_2$, namely, the P/O ratio was 1.68 and 2.54, respectively, and the corrected value for O_2 gas equilibrium was 1.01 and 1.40, respectively.

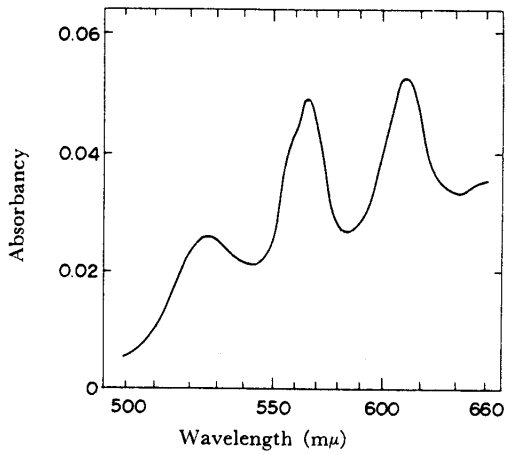


Fig. 4 Difference spectrum of a submitochondrial (R_{75-144}) fraction. A small amount of solid $Na_2S_2O_4$ was added as reducing agent.

Table 2 Component of cytochromes of submitochondrial particles
The concentrations of cytochromes were calculated from the difference spectra recorded with a multipurpose recording spectrophotometer (MPS-50, Shimazu Ltd.) and the extinction coefficients for cyt. a, ε mM = 16.0 (Δ605—Δ630 mμ); cyt. b, ε mM = 22.0 (Δ562—Δ575 mμ); cyt. c_1+c, ε mM = 19.1 (Δ553—Δ540 mμ).

	Cytochrome content (mμmole/mg protein)		
	cyt. a	cyt. b	cyt. c_1+c
HBHM	0.77	0.51	0.55
ETPH*	0.90	0.65	0.63

* ETPH; Standard submitochondrial fraction (R_{75-144}).

Table 3 Oxidase activity and P/O ratio of submitochondrial particles in the presence of succinate or $NADH_2$-generating system
Submitochondrial particles were prepared from HBHM stocked for 3 weeks at −20°. Assay was conducted as described in METHODS.

Substrate	Oxidase activity (mμmoles/min/mg protein)	P/O without correction*	P/O with correction*
Succinate	298	1.68	1.01
$NADH_2$	58	2.54	1.40

* The correction for O_2 gas equilibrium was conducted as described in METHODS.

Correlation of ATPase activity and capacity of oxidative phosphorylation of ETPH

The P/O ratios and ATPase activities in ETPH fractions obtained from the differential centrifugation are illustrated in Table 4. The ATPase activity in the hypotonic medium appeared to parallel with the capacity of oxidative phosphorylation of ETPH fractions. The ATPase of ETPH was sensitive to oligomycin.

Table 4 P/O ratios and ATPase activities in centrifugal fractions from sonicated heavy beef heart mitochondria

Fraction*	Relative P/O**	ATPase*** mμmoles/min/mg protein
R_{16-36}	0.28	76
R_{36-105}	1.00	202
$R_{105-144}$	1.10	213
$R_{144-198}$	0.29	106
Supernatant	0.00	107

* Abbreviation, R_{x-y}, was used as in Table 1.
** P/O ratio of each fraction was estimated in the presence of succinate and the relative P/O ratio was calculated as that of R_{36-105} was 1.00.
*** ATPase activity was assayed as described in the text.

Correlation of oxidative phosphorylation and membrane structure of ETPH

It is well known that the oxidative phosphorylation is uncoupled and the activity of oligomycin sensitive ATPase is enhanced by the hypotonic treatment of mitochondria or submitochondrial particles. The changes in

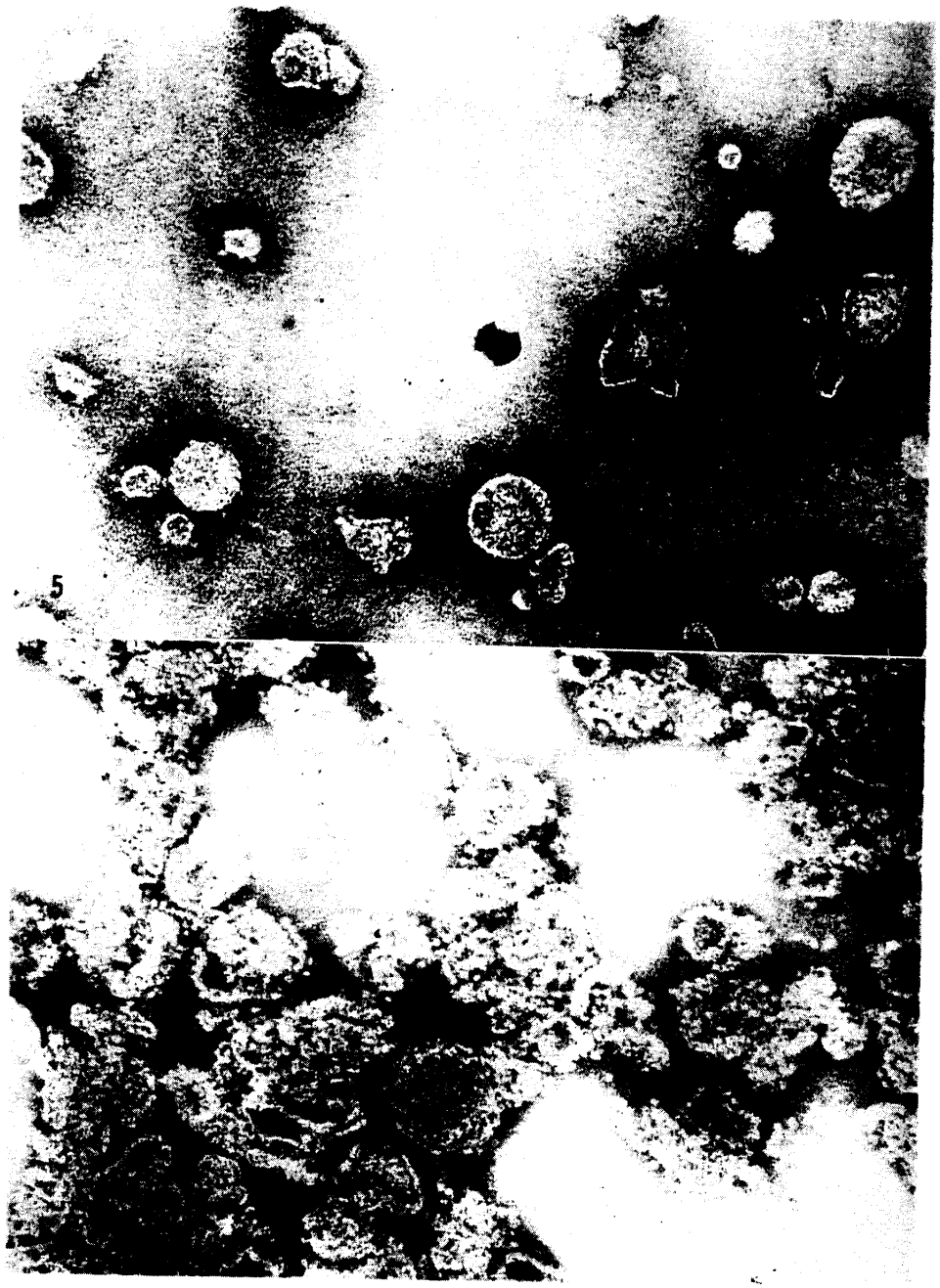
Table 5 Effect of hypotonic treatment on oxidative phosphorylation in submitochondrial particles

Reaction mixture was composed of 750 μmoles sucrose, 20 μmoles potassium phosphate (pH 7.4) 25 μmoles succinate, 5 μmoles $MgCl_2$, 0.55 ml of 1 mM Tris-HCl (pH 7.4) (or 0.55 ml of additions) and 2 mg protein of subm tochondrial particles in main chamber, and mixed with 5 μmoles ADP, 100 μmoles glucose and 200 K. M. units of hexokinase at the start of assay. Total volume of reaction mixture was 3.0 ml.

	Additions	Succinoxidase activity (mμatoms/min/mg protein)	P/O
ETPH		232	1.30
Hypo-ETPH (1 min)*		295	0.68
Hypo-ETPH (45 min)*		412	0.58
Hypo-w-ETPH**		457	0.69
Hypo-w-ETPH**	Supernatant**	536	0.34
ETPH		327	1.35
Hypo-w-ETPH**		325	0.54
Hypo-w-ETPH**	2mg BSA	325	0.48
Hypo-w-ETPH**	BSA+5μmoles ATP	288	1.03

* Hypo-ETPH (1 or 45 min); ETPH in stock medium were suspended in 9 volumes of 1 mM Tris buffer (pH 7.4) and stand for 1 or 45 min at 0° before the estimation of P/O.
** Hypo-w-ETPH and supernatant; ETPH in stock medium were suspended in 9 volumes of 1 mM Tris buffer (pH 7.4) and separated immediately the residue (Hypo-w-ETPH) from the supernatant by the centrifugation at 144,000×g for 30 min. At 45 min after initial suspension of ETPH, estimations of P/O were started.

the capacity of oxidative phosphorylation and in the membrane structure of ETPH after hypotonic treatment are shown in Table 5 and Figs. 5 and



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6. The P/O ratio of ETPH decreased to about 50 % by the hypotonic treatment for 1 to 45 minutes at 25° (Hypo-ETPH). The P/O ratio of this ETPH was restored about 40 % by the incubation with ATP and bovine serum albumin for 10 minutes at 30°. When the supernatant obtained by centrifugation of Hypo-ETPH was added to the submitochondrial particles (Hypo-w-ETPH), the P/O ratio decreased considerably and the oxygen uptake somewhat increased. ATPase activity or coupling factor was scarcely detected in this supernatant, and it rather suggested that an uncoupler was contained in the supernatant.

As shown in the Table 6, the capacity of oxidative phosphorylation of

Table 6 Effect of sonication on P/O ratio and ATPase activity of submitochondrial system

ETPH suspended in 0.25 M sucrose-10 mM Tris buffer, pH 7.5, (20 mg protein/ml) were sonicated for 90 sec per ml at maximum intensity (with 20 KC, 150 W, 7 ϕ tip), and separated immediatly the residue (S-ETPH) from the supernatant by centrifugation at 144,000×g for 30 min. The reaction mixture for oxidative phosphorylation contained 750 μmoles sucrose, 20 μmoles potassium phosphate (pH 7.5), 25 μmoles succinate, 5 μmoles $MgCl_2$ and sonicated ETPH (2.7 mg protein) or S-ETPH (2.5 mg protein) with or without addition of the supernatant (172 μg protein, equivalent amount) and mixed with 5 μmoles ADP, 100 μmoles glucose and 200 K. M. units of hexokinase.

	P/O	ATPase* (mμmoles/min/mg protein) −oligomycin	+oligomycin	% Inhibition
ETPH	0.98	264	25	90.5
Sonicated ETPH	0.59	327	46	85.9
S-ETPH	0.38	176	19	89.2
Supernatant	-	610	442	27.6
S-ETPH+supernatant	0.56	275	86	68.8

* The reaction mixture for the assay of ATPase activity contained 50 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$ and sonicated ETPH (675 μg protein)or S-ETPH (630 μg protein) and/or 4 times excess of supernatant (172 μg protein) with or without the addition of 2.5 μg oligomycin.

Fig. 5 Electron micrograph of the submitochondrial (R_{75-144}) fraction negatively stained with PTA in a slightly hypertonic condition. The (R_{75-144}) fraction was suspended in stock medium containing 0.25 M sucrose, 1 mM succinate, 1 mM ATP, 5 mM $MgCl_2$ and 10 mM $MnCl_2$ (pH 7.5) and mixed with an equal volume of 1.5 % PTA (pH 7.0) for 5 min, and an aliquot of the mixture negatively stained with 1 % PTA (pH 7.0). The surface structure appears to be compact and the head pieces and stalks of elementary particles are not so distinct. Magnification × 122,000

Fig. 6 Electron micrograph of submitochondrial (R_{75-144}) fraction negatively stained with PTA in a hypotonic condition. (R_{75-144}) fraction was suspended in 1 mM Tris buffer (pH 7.5) and stained negatively with PTA. The head pieces are clearly observed. Magnification ×122,000

ETPH decreased about 40 % with relatively severe sonication. After the sonicated ETPH were centrifuged at 144, 000×g for 30 minutes, the residue (S-ETPH) was separated from the supernatant fraction (S-Sap). The P/O ratio of S-ETPH decreased further about 20 %. The capacity of oxidative phosphorylation of S-ETPH was restored to the level before the separation on the addition of the S-Sap fraction. Oligomycin-insensitive ATPase was contained in the S-Sap fraction and the oligomycin-sensitivity also reappeared by the preincubation with the S-ETPH.

DISCUSSION

It is clear that the submitochondrial fraction showing the higher capacity of oxidative phosphorylation shows also higher activity of oligomycin sensitive ATPase. This fact supports the concept that ATPase integrated in submitochondrial membranes is an essential factor for the phosphorylation of ADP in oxidative phosphorylation, namely, in the terminal phosphorylation step.

After the separation of various submitochondrial fractions by the differential centrifugation following sonication of beef heart mitochondria, the smallest and lightest one having a significant capacity of oxidative phosphorylation was in the fraction sedimenting between 144, 000×g for 30 minutes and 198, 000×g for 60 minutes.

The submitochondrial particles appeared as being vesicular membrane structures by electron micrographs (cf. Figs. 2 and 3). When the submitochondrial particles were negatively stained in the isotonic medium, the surface structure of the submitochondrial particles appeared compact, and the head pieces of the elementary particles were not so distinct. On the other hand, by negative staining in hypotonic conditions, the surface of the submitochondrial particles gave a rough structural appearance and the head pieces and stalks of elemental particles could be clearly observed (cf. Figs. 5 and 6). The flexibility and conformational changes of the elementary particles of mitochondria were previously suggested by ODA and NISHI (26). It seems likely that these changes on the surface structure of the submitochondrial membranes correspond to those in the activity of the terminal step of oxidative phosphorylation. For instance, when the oxidative phosphorylation of the submitochondrial system is uncoupled by the treatment with hypotonic medium, the submitochondrial membrane appears to be in a swollen state with the projected head pieces.

There are many reports on the correlation between the functional state and the swelling-contraction of mitochondria. One of them describes

about the high-amplitude swelling and contraction of mitochondria (27), and the other about the low-amplitude structural changes of mitochondria (28). When the submitochondrial particles were treated with hypotonic medium, the membrane structures were modified and in this instance the P/O ratio of the submitochondrial particles decreased considerably. The capacity of oxidative phosphorylation, however, could be restored by the preincubation with ATP and bovine serum albumin. These facts suggest that the response of the submitochondrial particles to the hypotonic treatment may be similar to that of the high amplitude swelling and contraction in mitochondria.

A coupling factor having the activity of oligomycin-insensitive ATPase was released into the supernatant after the sonication of the submitochondrial particles, and it could be similar to the coupling factor 1 (F_1) found by RACKER *et al.* (18, 19). It is clearly suggested that one of the essential factors for the submitochondrial system to have the capacity of oxidative phosphorylation is to have the oligomycin-sensitivity.

The responses in the terminal phosphorylation step and in the conformational changes of the submitochondrial particles to a simple physicochemichal treatment may be relatively well analyzed, but those on the more complicated treatments cannot be so simple. For example, the analysis of the mechanism of oxidative phosphorylation in "AS" particles (30) obtained with the filtration of the submitochondrial particles through the Sephadex colum is considerably complicated. Although several coupling factors in addition to F_1 have been reported (30—34), further investigations are necessary to analyze the relation between the function of these factors and the structure of the inner membranes of mitochondria.

SUMMARY

1. After the centrifugation of sonicated heavy beef heart mitochondria at 75, 000×g for 10 minutes, the supernatant was centrifuged at 144, 000×g for 30 minutes. The residue was revealed being composed of vesicular inner membrane fragments (ETPH), about 600 to 1000 Å in diameter, showing a morphological homogeneity and a high capacity of oxidative phosphorylation.

2. The P/O ratio of the ETPH in the presence of succinate and of $NADH_2$ was 1.68 and 2.54, respectively, and the corrected P/O value for O_2 gas equilibrium was 1.01 and 1.40, respectively.

3. The capacity of oxidative phosphorylation in ETPH fraction was parallel to the activity of the oligomycin- sensitive ATPase in these fractions.

4. The P/O ratio of ETPH was decreased to about 50 % by hypotonic treatment. The decrease of P/O ratio was restored to the level of about 90 % by incubating the ETPH with ATP and BSA. In the instance where the P/O ratio was low level in the hypotonic medium, the surface structure of ETPH was observed as a swollen form and the head pieces of the elementary particles were clearly observed in contrast to the solid surface structure of ETPH in the isotonic medium.

5. The P/O ratio of ETPH was decreased to about 60 % by relatively severe sonication, and after separating the residue from the supernatant, that of the residue decreased further to about 40 %. The P/O ratio of the residue was restored to the level before the separation on the addition of the supernatant containing oligomycin-insensitive ATPase.

6. A discussion was made on the correlation between the surface structure and the activities at terminal phosphorylation step of ETPH after the simple physico-chemical treatment.

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