Nagoya J. Med. Sci. **54.** xx ~ xx, 1992

ISOLATION AND SOME BIOCHEMICAL PROPERTIES OF PORCINE PANCREAS MITOCHONDRIA

YUZO SAKAI[1], KAYO ADACHI[2], TETSUO HAYAKAWA[1] and TAKASHI WAKABAYASHI[2]

[1]2nd Department of Internal Medicine and [2]Department of Anatomy, Nagoya University School of Medicine, Nagoya 466, Japan

ABSTRACT

Morphologically and functionally intact mitochondria were isolated from the porcine pancreas using a conventional, differential centrifugation method. The homogenate of the porcine pancreas made in a medium containing 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 0.5% bovine serum albumin and 1 mM EDTA, in the presence or in the absence of dibucaine and trypsin inhibitor, was centrifuged for 10 min at 700x*g*. The supernatant was centrifuged for 10 min at 7000x*g*, and the pellet was washed three times. Trypsin activity of mitochondria isolated in the absence of dibucaine and trypsin inhibitor was as low as that of mitochondria isolated in the presence of dibucaine and trypsin inhibitor, and a major part of the activity remained inactivated. Phospholipase A_2 activity of the former mitochondria was as low as that of the latter, and remained unchanged up to 8–10 hr at 4°C. The presence of bovine serum albumin and EDTA in the respiration medium was absolutely required to obtain good respiratory controls of those mitochondria. These data suggest that well-coupled mitochondria can be obtained from the pancreas by a conventional isolation procedure without activating the major part of trypsin.

Key Words: Pancreas; Mitochondria; Dibucaine; Trypsin inhibitor; Phospholipase A_2; Bovine serum albumin

INTRODUCTION

The role of mitochondria in the pathogenesis of various pancreatic diseases largely remains to be solved. Data to correlate abnormal mitochondrial functions to pancreatic pathologic conditions are scanty. It has been suggested that abnormal mitochondrial functions may account for alcoholic fatty liver[1)] or alcoholic pancreatic steatosis[2)], although controversial data have been presented by other[3)]. In necrotizing pancreatitis induced experimentally by the administration of lysine, the earliest change in acinar cells is the swelling of mitochondria[4)]. Taking into account the central role of mitochondria in the energy metabolism of the cell, it is reasonable to assume that disturbances in the function of mitochondria are directly or indirectly related to various pathologic states of the pancreas. Thus, it is urgent to establish the isolation procedure for pancreas mitochondia. However, a survey of the literature has revealed that data on pancreas mitochondria are surprisingly few. Sardesai *et al.*[5)] could not observe oxygen uptake of mitochondria isolated from the rat pancreas, and suggested the presence of some inhibitory substance on mitochondrial respiration. Recently, Wilson *et al.*[3)] have developed a quick method for the isolation of intact mitochondria from the rat pancreas. Using their method, they obtained ADP/O ratios of 2.6 ± 0.1 and 1.4 ± 0.2 when oxidizable substrates were glutamate (+ malate) and succinate, respectively. The isolation medium they used contained dibucaine to inhibit

Correspondence: Dr. Takashi Wakabayashi, Department of Anatomy, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan

Received for Publication in March 14, 1991

phospholipase A_2 activity, and soybean trypsin inhibitor to prevent the activation of trypsinogen.

In the present study we demonstrate that tightly coupled pancreas mitochondria can be obtained by a routine isolation procedure for liver mitochondria without using dibucaine and trypsin inhibitor. Instead, bovine serum albumin (BSA) and EDTA are absolute requirements for the isolation of intact mitochondria from the pancreas.

MATERIALS AND METHODS

Isolation of mitochondria from porcine pancreas

Porcine pancreatic glands obtained from a slaughter house within one hr after the death of the animal were trimmed of covering fat and finely minced with a single-edged razor blade. They were homogenized in a medium containing 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.5% fatty acid free bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis), 0.25 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis) and 400 μM dibucaine according to the method of Wilson *et al.*[3]. The homogenization of the tissues was carried out in a Teflon-glass homogenizer using a Labo-stirrer (type LR-51B, Yamato Scientific Co., Tokyo) with five up-and-down strokes at 800 rpm. In the control experiments, either dibucaine or trypsin inhibitor or both were omitted from the medium described above. Furthermore, a routinely used isolation medium for liver mitochondria in our laboratory was also used in some experiments. The medium contained 2 mM Hepes, pH 7.4, 70 mM sucrose, 220 mM mannitol, 0.05% BSA and 0.1 mM EDTA. The mitochondiral fraction was obtained by the following two differential centrifugation methods: 1) The method of Wilson *et al.*[3]: The homogenate made in various isolation media, described above, was centrifuged at 2000x*g* for 7 min. The residue consisted of two layers. A thin, surface layer was pale brown indicating that a part of the mitochondria were sedimented. The inner layer of the residue was whitish indicating that the zymogen granules were largely sedimented in addition to nuclei and cell debris. The resultant supernatant was centrifuged at 6000x*g* for 10 min to sediment mitochondria. The pellet thus obtained was again composed of two layers. The upper layer was brownish and the lower layer was whitish. The upper layer was collected carefully leaving the lower layer to avoid the contamination of zymogen granules, and it was suspended in a small amount of the medium. This is the mitochondrial fraction that Wilson *et al.*[3] developed, and it will be designated as the 2000—6000x$g \cdot R_2$ fraction hereafter; 2)The homogenated made in various isolation media was centrifuged at 700x*g* for 10 min. The supernatant thus obtained was centrifuged at 7000x*g* for 10 min to sediment mitochondria. This will be designated as the 700—7000x$g \cdot R_2$ fraction hereafter. The R_2 fraction, obtained either by the 2000—6000x*g* centrifugation method or by the 700—7000x*g* centrifugation method, was washed three times at the same speed at which it was sedimented. The fraction thus obtained was designated as the 2000—6000x$g \cdot R_2'''$ or the 700—7000x$g \cdot R_2'''$ fraction. Protein concentrations were determined by the method of Lowry *et al.*[6].

Measurement of oxygen uptake

Respiration of mitochondria was measured in the medium described by Wilson *et al.*[3]. Namely, the medium contained 250 mM sucrose, 22 mM KCl, 22 mM triethanolamine, pH 7.4, 1 mM EDTA, and 0.5% fatty acid free BSA (medium A). In some experiments, the respiration medium routinely used in our laboratory was also tested. It contained 70 mM sucrose, 220 mM mannitol, 5 mM Tris-Cl, pH 7.4 and 0.1 mM EDTA (medium B). The rate of oxygen

consumption was measured at 30°C with a Clark-type oxygen electrode (Beckman Co., Fullerton, California). Oxidizable substrates were 5 mM glutamate (+ 5 mM malate) or 5 mM succinate. State 3 respiration was measured by the addition of ADP 200–300 nmoles to the reaction mixture in the presence of substrate and Pi (5 mM). State 4 respiration was defined as the respiratory rate after the expenditure of ADP. ADP/O ratios and respiratory control index (RCI) were calculated according to the method of Estabrook[7].

Enzyme assays

Cytochrome oxidase (EC 1.9.3.1) activity was measured using a Clark-type electrode as described before[8]. The reaction medium contained 10 mM Tris-Cl, pH 7.4, 2 mM ascorbate and cytochrome *c* (0.5 mg/ml) (type IV, from horse heart, Sigma Chemical Co., St. Louis). Ascorbate was prepared freshly prior to use. Lysophosphatidylcholine (1–2 mg/mg mitochondrial protein) was added to the reaction mixture as a solubilizing agent to obtain full activity. The rate of oxygen consumption during the oxidation of reduced cytochrome c in the presence of ascorbate indicated the cytochrome oxidase activity.

Mg^{2+}-activated ATPase (EC 3.6.1.3): The activity of mitochondria was measured in the presence of 2,4-dinitrophenol[9]. The activity was measured in a medium containing 250 mM sucrose, 10 mM Tri-Cl, pH 7.4, 3 mM $MgCl_2$ and 0.1 mM EDTA. A 100 μl portion of 100 mM ATP (pH 7.4) was added to 900 ul of the medium containing 0.50 mg of mitochondria at 30°C. After 10 min, 1 ml of 16% perchloric acid was added to the incubation mixture. The reaction mixture was then centrifuged at 8000x*g* for 10 min. The inorganic phosphate released to the supernatant was spectrophotometrically measured according to the method of Fiske and Subbarow[10] as described before[9].

Trypsin activity of tissue homogenate or mitochondria was determined spectrophotometrically by the method of Hayakawa *et al.*[11] using α-N/benzoyl-DL-arginine p-nitroanilide HCl (BAPNA) (Sigma Chemical. Co., St. Louis) as the substrate with or without activation by enterokinase. Aqueous stock solution of BAPNA (1 mg/ml) was prepared by heating to 85°C and then cooling on ice. It was diluted at equal volumes with 0.1 M Tris-Cl, pH 8.0, containing 0.04 M $CaCl_2$ just prior to use. Trypsin (2x crystallized, sigma) was dissolved in 0.0025 N HCl as a stock solution (1 mg/ml). To obtain a standard curve for trypsin, the stock solution of trypsin was diluted to appropriate concentrations with 0.025 N HCl containing BSA at a concentration of 1 mg/ml. Enterokinase (EC 3.4.21.9) (Sigma) was dissolved in 0.2 M Tris-Cl, pH 8.0 at a concentration of 10 mg/ml. Protein concentration of a mitochondrial fraction or homogenate was brougth to 5–10 mg/ml with 0.2 M Tris-Cl, pH 8.0. A 10 μl portion of the sample was put into test and reference tubes containing 2 ml of BAPNA solution (0.5 mg/ml) and 2ml of acetic acid (30%, v/v), respectively. They were incubated for 10 min at 37°C, and then a 2 ml portion of acetic acid and a 2 ml portion of BAPNA solution were put into test and reference cuvettes, respectively. Difference in absorbance between the test and the reference cuvettes was read at 410 nm using Hitachi Model 100–40 spectrophotometer. Similarly, the standard curve for trypsin was obtained using BAPNA. Trypsin activity of the sample was calculated from absorbance changes of the sample and the trypsin standard, and expressed as ug trypsin/mg protein. To activate trypsinogen by enterokinase, an equal volume of a solution of enterokinase was added to a sample and the mixture was incubated for 1 hr at 4°C. A 10 μl portion of the mixture was withdrawn, and trypsin activity was measured as described above.

Phospholipase A_2 (EC 3.1.1.4) activity of a mitochondrial fraction or homogenate was measured essentially according to the method of Tamiya-Koizumi *et al.*[12] The homogenate (0.2–0.7 μg protein) or mitochondria (0.4–3.0 μg protein) was incubated for 60 min at 37°C with 3 nmoles of 1-acyl-2-(1-^{14}C) arachidonyl phosphatidylethanolamine (specific activity,

10,000 cpm/nmol) in a 200-μl system containting 100 mM Tris-acetate, pH 9.0 and 10 mM $CaCl_2$. The reaction was terminated by adding 4 μl of acetic acid and 800 μl of chloroform/methanol (2:1, v/v) containing 20 μg of arachidonic acid. The reaction mixture was centrifuged, and the lower phase of the supernatant was collected and concentrated. The concentrated sample was then chromatographed on plastic sheets of silica gel 60 (Kanto Chemical Co., Tokyo) with chloroform/methanol/water (65:25:3, v/v/v/). The spot corresponding to arachidonic acid was cut out and its radioactivity was measured using a toluene scintillator with a liquid scintillation spectrometer. The reactions were linear at concentrations of protein ranging 0.1—1.0 μg and 0.4—3.0 μg for homogenate and mitochondria, respectively.

ATP synthesis

ATP synthesis of mitochondria was measured by the luciferin-luciferase method[13] as described before[14]. This method of ATP analysis is based on the linear luminescence response of firefly luciferin to added ATP. Luciferin is converted into oxyluciferin by luciferase in the presence of ATP, Mg^{2+} and oxygen. Light generated during the reaction is measured with a photomultiplier Mitochondria were preincubated for 10 min at 30°C with 5 ml of medium A (1.2 mg/ml). Then, 5 mM sodium glutamate, 5 mM sodium malate, 5 mM potassium phosphate, and 500 nmoles of ADP were added to the reaction mixture. After 0, 0.5, 1, 1.5 and 2 min, a 20 μl portion of the sample was withdrawn and put into a test tube containing 980 μl of the ice-cold medium A. The sample (100 μl) was immediately put into a cuvette containing 100 μl of ATP releasing agent (Labo Science, Tokyo). The cuvette was mounted into a TD-4000 lumiphotometer (Labo Science, Tokyo) and a 100-μl portion of luciferin-luciferase (EC 1.14.14.3) (Labo Science, Tokyo) was added to the cuvette. To correlate light intensity with the concentration of ATP, light intensity was plotted against standard ATP solutions (1×10^{-7}—2.5×10^{-6} M). The light intensity occurring after the addition of luciferin-luciferase to the cuvette containing an unknown concentration of ATP was thus related to the concentration of ATP.

Cytochrome contents

Cytochrome contents of mitochondria were measured spectrophotometrically according to the method of Williams[15] as described before[16]. Mitochondria (10 mg) dissolved in 900 μl of 100 mM phosphate buffer, pH 7.4, containing 2.2% deoxycholic acid were put into two cuvettes, respectively. A 100 μl portion of 0.05 M $K_3Fe(CN)_6$ as the oxidizing agent was added to one cuvette, and 100 μl of 50 mM ascorbate as the reducing agent was added to the other cuvette. A few grains of sodium hydrosulfite were put into the latter cuvette. The reduced-oxidized difference visible spectra of the cytochromes were obtained using a Hitachi U-3200 spectrphotometer.

Electron microscopy

Mitochondrial suspensions were fixed in 2% glutaraldehyde containing 250 mM sucrose and 50 mM sodium cacodylate, pH 7.4, and processed for electron microscopy as described before[17]. Thin sections were cut on a Reichert ULTRACUT N ultramicrotome, stained with lead citrate and examined in a Hitachi H-800 electron microscope operated at 100 kV.

RESULTS

Yields of mitochondrial proteins obtained by various isolation procedures

When the 2000—6000x*g* R_2 fraction obtained in the presence (the mitochondrial fraction of

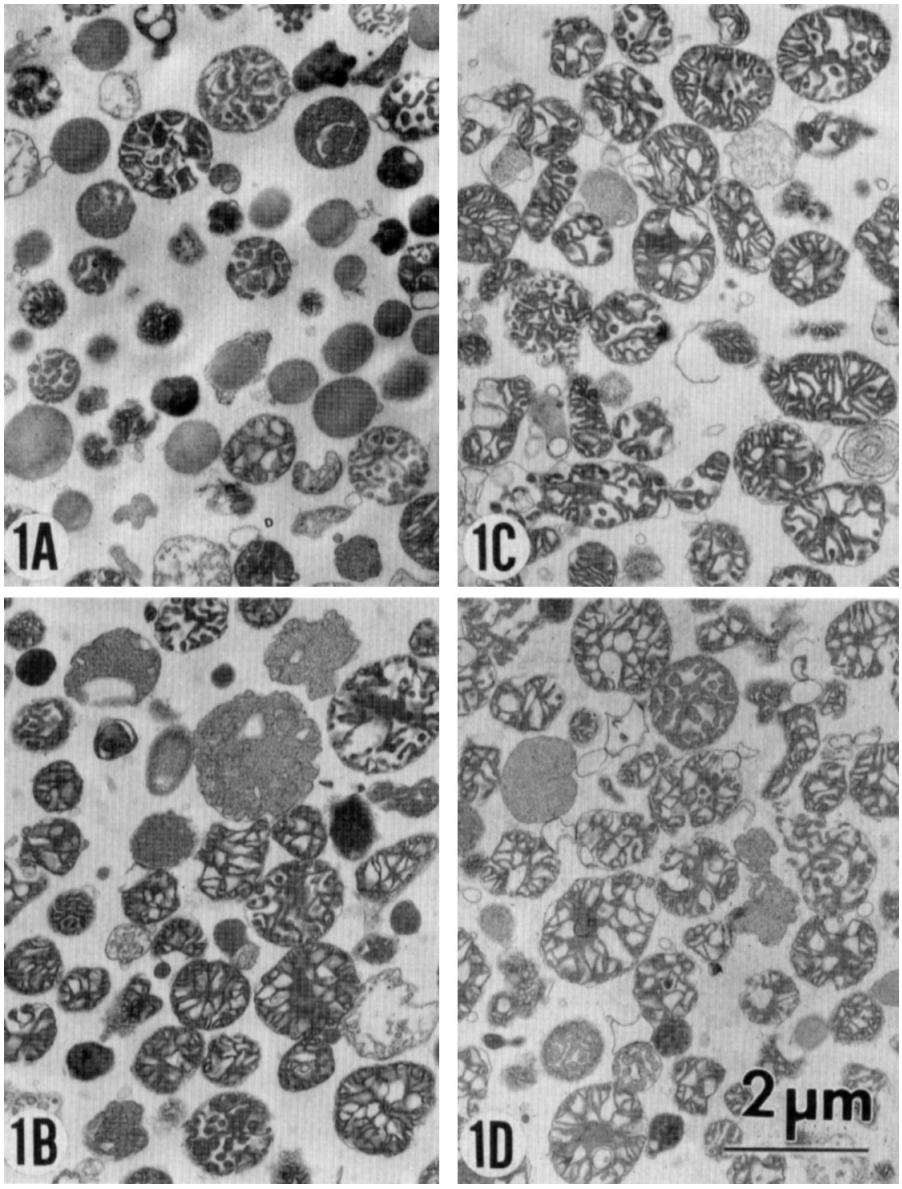


Fig. 1 Electron micrographs of pancreatic mitochondrial fractions obtained by various isolation procedures. For details, see Materials and Methods.

A. A 2000–6000x$g \cdot R_2$ fraction isolated in the presence of dibucaine and trypsin inhibitor. ×13700.

B. A 2000–6000x$g \cdot R''_2$ fraction isolated in the presence of dibucaine and trypsin inhibitor. ×13700.

C. A 700–7000x$g \cdot R''_2$ fraction isolated in the presence of dibucaine and trypsin inhibitor. ×13700.

D. A 700–7000x$g \cdot R''_2$ fraction isolated in the absence of dibucaine and trypsin inhibitor. ×13700.

Wilson *et al.*[3]) or absence of dibucaine and trypsin inhibitor was washed three times, the yield of the 2000–6000x*g* · R'''_2 fraction thus obtained to the homogenate was decreased to about 30% of that of the 2000–6000x*g* · R_2 fraction to the homogenate. As was expected, the yield of the 700–7000x*g* · R_2 fraction obtained in the presence or absence of dibucaine and trypsin inhibitor to the homogenate was larger than that of the 2000–6000x*g* · R_2 fraction to the homogenate. When the homogenate was centrifuged either at 2000x*g* for 7 min or at 700x*g* for 10 min, the residue was made of two layers: an upper layer, which was pale brown and relatively rich in mitochondria, and a lower layer, which was whitish and rich in zymogen granules. The two layers were not well demarcated. In the present study special care was taken to collect only the upper layer leaving the whitish layer attached to the bottom of the centrifuge tube. The R_2 fraction thus obtained by the 2000–6000x*g* centrifugation method was the mitochondrial fraction used by Wilson *et al.*[3] (Fig. 1A). The higher yield of R_2 fraction obtained by the 700–7000x*g* centrifugation method than by the 2000–6000x*g* centrifugation method was, as expected, due to severer contaminations with zymogen granules and membrane fragments mainly composed of endoplasmic reticulum. When the R_2 fraction obtained by either 2000–6000x*g* or 700–7000x*g* was washed three times, a fraction rich in mitochondria (R'''_2 fraction) was obtained (Figs. 1B–1D). The yield of the R'''_2 fraction to the homogenate obtained by the 700–7000x*g* centrifugation method tended to be slightly higher than that of the R'''_2 fraction obtained by the 2000–6000x*g* centrifugation method (Table 1). The yield of the pancreatic mitochondrial fraction (R'''_2) to the homogenate or the tissue was extremely low when it was compared with that of the liver. On the other hand, a ratio of the homogenate to the wet weight of the pancreas based on the amount of protein was larger than that of the liver. One of the reasons for this may be a smaller population of mitochondria per one acinar cell compared with that of the hepatic parenchymal cell since pancreatic acinar cells are occupied by abundant rough-surfaced endoplasmic reticulum and zymogen granules. Another possible reason may be that porcine pancreas is rich

Table 1. Yield of pancreatic mitochondrial proteins obtained by various isolation procedures[a,b]

	2000–6000x*g*[c]		700-7000x*g*[d]		Liver
	+Dib., +T.I.	−Dib., −T.I.	+Dib., +T.I.	−Dib., −T.I.	
R_2/Homogenate (%)	4.55 ± 0.28	4.51 ± 0.03	5.77 ± 0.38*	5.96 ± 0.34*	
R_2/Wet weight of the tissue (%)	1.14 ± 0.07	1.09 ± 0.08	1.44 ± 0.11*	1.47 ± 0.09*	
R'''_2/Homogenate (%)	1.32 ± 0.22	1.39 ± 0.12	1.56 ± 0.36	1.65 ± 0.31	8.16 ± 0.21
R'''_2/Wet weight of the tissue (%)	0.33 ± 0.05	0.34 ± 0.03	0.39 ± 0.08	0.40 ± 0.06	1.61 ± 0.09
Homogenate/Wet weight of the tissue (%)	25.04 ± 1.25	24.21 ± 0.82	24.92 ± 0.87	24.71 ± 1.16	20.03 ± 0.63

a Basic isolation medium contained 10 mM Tris-Cl, pH7.4, 0.25M sucrose, 1 mM EDTA, 0.5% BSA in the presence or absence of 0.25 mg/ml soybean trypsin inhibitor (T.I.) and 400 uM dibucaine (Dib.).

b Data are the average of four different experiments (mean ± SE). *Statistically differnet from the corresponding data obtained by 200–6000x*g* ($0.02 < p < 0.05$).

c Homogenate was centrifuged at 2000x*g* for 7 min. The resultant supernatant was centrifuged at 6000x*g* for 10 min to sediment the crude mitochondrial pellet (R_2). The R_2 fraction was obtained by washing R_2 fraction three times.

d Homogenate was centrifuged at 700 x*g* for 10 min. The resultant supernatant was centrifuged at 7000x*g* for 10 min. The three-time-washed R_2 fraction was designated as the R_2 fraction.

Table 2. Contents of cytochromes ion pancreas mitochondria obtained by various isolation procedures[a]

Centrifugation Method	Fraction	Cytochromes (nmol/mg protein)				
		$a+a_3$	b	c_1	c	$c+c_1$
2000−6000xg	R_2	0.133±0.004	0.127±0.011	0.073±0.012	0.054±0.004	0.127±0.015
2000−6000xg	R''_2	0.207±0.016	0.171±0.012	0.088±0.007	0.079±0.006	0.167±0.010
700−7000xg	R_2	0.124±0.012	0.118±0.013	0.054±0.003	0.056±0.004	0.110±0.005
700−7000xg	R''_2	0.270±0.010*	0.218±0.012**	0.076±0.013	0.089±0.005	0.164±0.016
Liver	R''_2	0.196±0.004	0.190±0.006	0.128±0.004	0.191±0.010	0.324±0.014

a Homogenate of porcine pancreas was centrifuged either for 7 min at 2000xg or for 10 min at 700xg. The supernatant was centrifuged for 10 min either at 6000xg or at 7000xg. The residue (R_2) was washed three times (R''_2). The isolation medium contained 10 mM Tris-Cl, pH 7.4, 0.25M sucrose, 1 mM EDTA, 0.5% BSA, soybean trypsin inhibitor (0.25 mg/ml) and 400 μM dibucaine. Data are the average of six different experiments (mean±SE). Comparison was made between 2000−6000xg·R_2 and 700−7000xg·R_2, and between 2000−6000xg·R''_2 and 700−7000xg·R''_2.
*$0.001 < p < 0.01$; **$0.01 < p < 0.02$

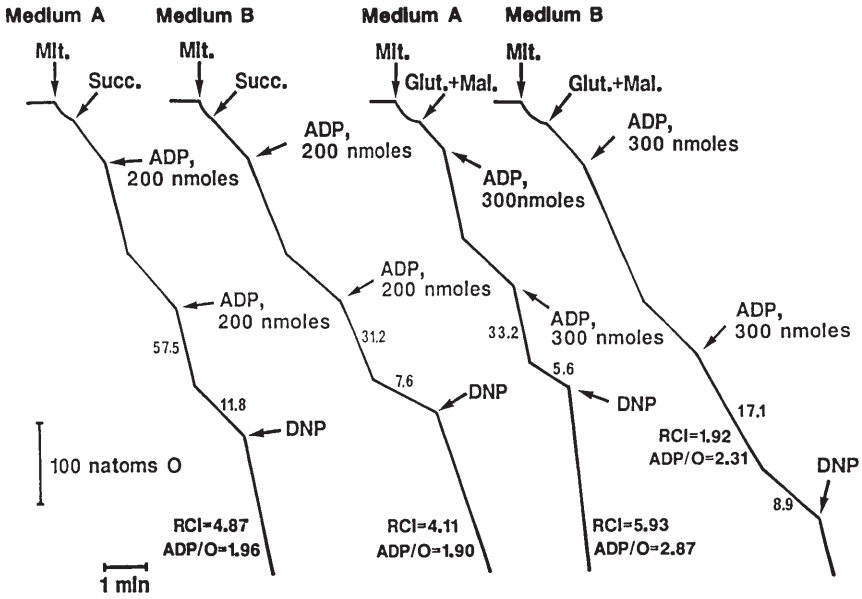
both in intra- and interlobular fibrous connective tissue lowering the ratio of parenchymatous acinar cells to the homogenate or the tissue.

Contents of cytochromes in pancreatic mitochondria

There was statistically no difference in the content of cytochromes between the 2000−6000xg·R_2 fraction and the 700−7000xg·R_2 fraction. However, the content of cytochromes in the latter fraction became much larger than that in the former fraction after both fractions were washed three times (Table 2). This was due to remarkable increases in cytochromes $a+a_3$ and b in the latter fraction. The content of cytochromes $a+a_3$ and b in the 700−7000xg·R''_2 fraction was higher than that of rat liver mitochondria whereas that of cytochrome $c+c_1$ was approximately half of the latter mitochondria.

Coupling efficiency of pancreas mitochondria obtained by various isolation procedures

Before we measured the coupling efficiency of mitochondria obtained by various isolation procedures, we compared two different respiration media: medium A, according to Wilson *et al.*[3]; and medium B, routinely used in our laboratory for liver mitochondria. In Fig. 2, typical examples of oxygen traces of a 700−7000xg·R_2 fraction obtained in the above-described two different media are shown. The rate of oxygen consumption in state 3 respiration after the addition of ADP was definitely lower in medium B than in medium A resulting in lower repiratory control in the former especially when glutamate was the oxidizable substrate. Thus, we employed medium A as a respiration medium thereafter. However, we found later that medium A could be replaced by medium B as long as the concentrations of BSA and EDTA of the latter were as high as those of the former. The rates of oxygen consumption both in state 3 and state 4 (after the conversion of externally added ADP to ATP) respiration were low in the 2000−6000xg·R2 fraction compared with those in the 2000−6000xg·R''_2 fraction or in the 700−7000xg·R''_2 fraction (Table 3). Lower rates of oxygen consumption of the R_2 fraction corresponded well to the fact that the fraction was severely contaminated with zymogen granules



Fug. 2. Rate of oxygen consumption of pancreas mitochondria measured in different respiration media. The rate of oxygen consumption of mitochondria was measured at 30°C in mediums A and B, respectively, using a 700–7000x*g* · R_2 fraction isolated in the presence of dibucaine and trypsin inhibitor. Final concentrations of reagents added were Pi, 5 mM; succinate, 5 mM; glutamate, 5 mM; malate, 5 mM; and DNP, 25 μM. Numbers in the figures indicate the amount of oxygen (natoms) consumed/min/mg protein. Medium A contained 250 mM sucrose, 22 mM KCl, 22 mM triethanolamine, pH 7.4, 1 mM EDTA and 0.5% BSA. Medium B contained 70 mM sucrose, 220 mM mannitol, 5 mM Tris-Cl, pH 7.4, and 0.1 mM EDTA.

Table 3. Coupling efficiency of porcine pancreas mitochondria obtained by various isolation procedures[a]

	Fraction	Centrifugation	Isolation Medium	State 3 (natoms O/mg/min)	State 4 (natoms O/mg/min)	RCI	ADP/O ratio
Succinate	R_2	2000–6000x*g*	STBE+Dib.+T.I.	58.9 ± 12.0	13.3 ± 1.8	4.36 ± 0.34	1.70 ± 0.24
	R''_2	2000–6000x*g*	STBE+Dib.+T.I.	89.0 ± 11.9	20.4 ± 5.2	4.70 ± 1.05	2.08 ± 0.11*
	R''_2	700–7000x*g*	STBE+Dib.+T.I.	93.7 ± 8.8*	21.7 ± 3.9*	4.50 ± 0.82	1.91 ± 0.19
	R''_2	700–7000x*g*	STBE–Dib.–T.I.	91.4 ± 12.4*	20.6 ± 2.6*	4.58 ± 1.00	1.92 ± 0.14
Glutamate (+Malate)	R_2	2000–6000x*g*	STBE+Dib.+T.I.	40.6 ± 9.7	8.3 ± 1.0	4.13 ± 0.44	2.86 ± 0.16
	R''_2	2000–6000x*g*	STBE+Dib.+T.I.	62.3 ± 6.5	10.7 ± 3.3	6.60 ± 1.91	2.90 ± 0.17
	R''_2	700–7000x*g*	STBE+Dib.+T.I.	69.0 ± 6.0*	14.1 ± 3.1*	5.12 ± 0.79	2.96 ± 0.08
	R''_2	700–7000x*g*	STBE–Dib.–T.I.	70.1 ± 3.4*	16.6 ± 2.7*	4.36 ± 0.61	3.00 ± 0.16

a Porcine pancreas was homogenized in a medium containing 10 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 0.5% BSA and 1 mM EDTA (STBE) in the presence or absence of 400 μM dibucaine (Dib.) and 0.25 mg/ml tryspin inhibitor (T.I.). The homogenate was centrifuged for 7 min at 2000x*g* or for 10 min at 700x*g*. The resultant pellet was suspended in the appropriate medium, and centrifuged for 10 min at 6000x*g* or 7000x*g*. The pellet (R_2) was washed three times (R''_2). Data are the average of three different experiments (mean ± SE).

* Statistically different from data obtained by the 2000–6000x*g* · R_2 fraction at $0.02 < p < 0.05$.

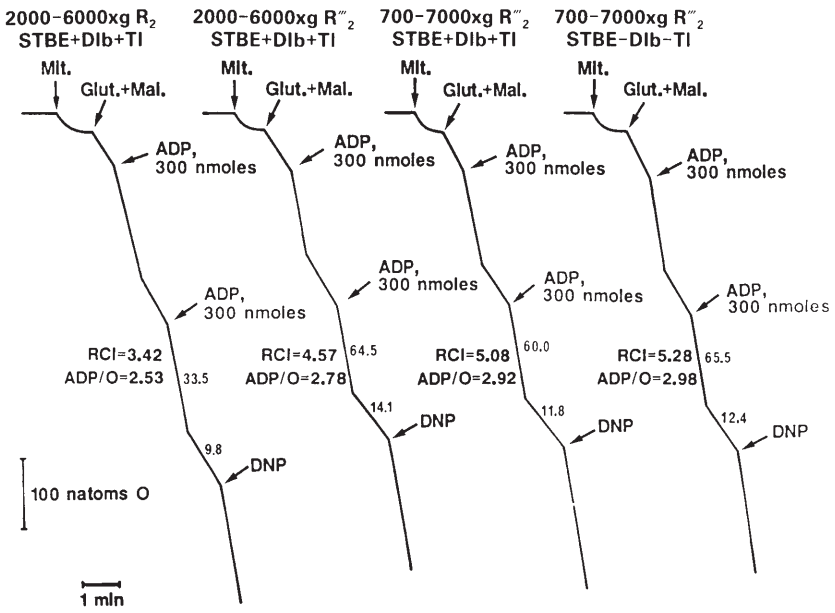


Fig. 3. Coupling efficiency of pancreatic mitochondrial fractions obtained by various isolation procedures. The respiration of mitochondria was measured in medium A. Isolation medium contained 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA and 0.5% BSA (STBE) in the presence and absence of 400 μM dibucaine (Dib.) and 0.25 mg/ml soybean trypsin inhibitor (T.I.).

and microsomes as shown in the previous section. Another point to be stressed here is the effect of the composition of the isolation medium on the coupling efficiency of those mitochondria. The coupling efficiency of mitochondria obtained in the absence of both dibucaine and trypsin inhibitor in the isolation medium was unexpectedly as good as that of mitochondria isolated in the presence of both dibucaine and trypsin inhibitor. Typical examples of oxygen traces of such mitochondria are shown in Fig. 3. Moreover, the coupling efficiency of mitochondria isolated in the absence of dibucaine and trypsin inhibitor was well maintained for at least 8–10 hr after they were isolated. A tightly coupled mitochondrial fraction was obtained also from the rat pancreas using a medium not containing dibucaine and trypsin inhibitor. These results strongly suggest that enzymes of zymogen granules including trypsinogen are not easily activated during the isolation procedure of pancreas mitochondria. Data on trypsin and phospholipase A_2 activities of pancreas mitochondria will be presented later.

ATPase and cytochrome oxidase activities of pancreas mitochondria obtained by various isolation procedures

In the previous section we have shown that mitochondria isolated in the absence of dibucaine and trypsin inhibitor are well coupled. To analyze further the effect of the composition of isolation medium on mitochondrial functions, we also measured ATPase and cytochrome oxidase activities of those mitochondria (Table 4). It is clear from the table that the absence of dibucaine and trypsin inhibior did not affect the activities of either enzyme.

Table 4. Cytochrome oxidase and ATPase activities of pancreas mitochondria obtained by various isolation procedures[a]

Centrifugation	Isolation Medium	Fraction[b]	Cytochrome oxidase (natoms O/mg/min)	ATPase[c] (nmol Pi/mg/min)
2000–6000x*g*	STBE+Dib.+T.I.	R_2	109.8 ± 5.0	99.2 ± 2.0
2000–6000x*g*	STBE+Dib.+T.I.	R''_2	338.0 ± 27.0*	191.2 ± 8.6*
700–7000x*g*	STBE+Dib.+T.I.	R''_2	356.9 ± 11.9*	254.2 ± 17.8*
700–7000x*g*	STBE–Dib.–T.I.	R''_2	353.7 ± 6.0*	261.4 ± 11.2*

a Isolation medium for porcine pancreas contained 10 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.5% BSA (STBE) in the presence or absence of 400 μM dibucaine (Dib.) and 0.25 mg/ml soybean trypsin inhibitor (T.I.). Data are the average of three different experiments (mean ± SE).

b The crude mitochondrial fraction (R_2) was washed three times either at 6000x*g* or at 7000x*g* (R''_2).

c Mg^{2+}-activated ATPase activity was measured using frozen samples in the presence of DNP to obtain full activity.

* Statistically different from the 2000–6000x*g* · R_2 fraction at $p < 0.001$.

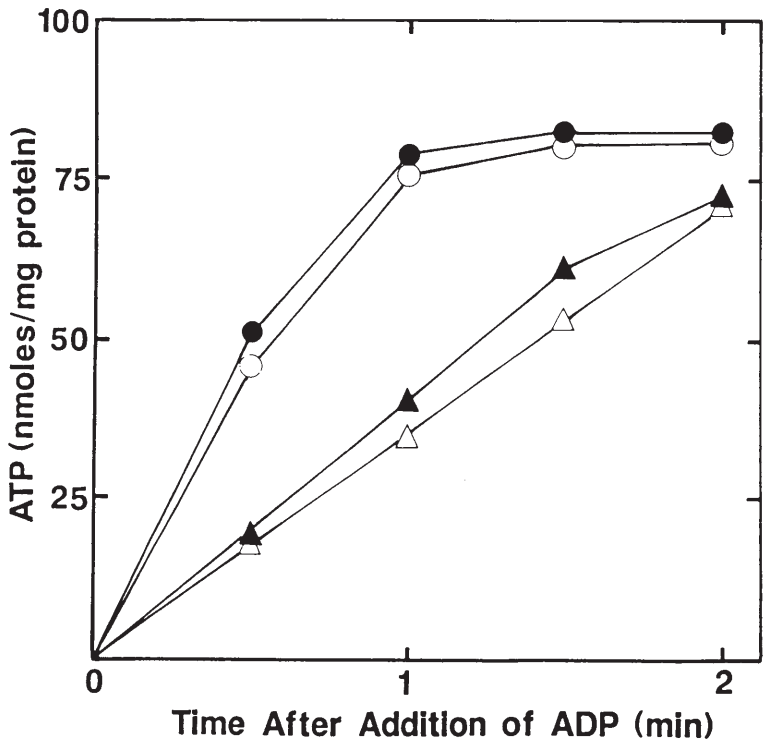


Fig. 4. Ability of pancreatic mitochondrial fractions obtained by various isolation procedures to synthesize ATP. For details, see Materials and Methods. A 2000–6000x*g* · R_2 fraction isolated in a medium containing 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.5% BSA in the presence (△) or the absence (▲) of 400 μM dibucaine and 0.25 mg/ml soybean trypsin inhibitor; a 700–7000x*g* · R''_2 fraction isolated in a medium containing 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.5% BSA in the presence (○) or absence (●) of 400 μM dibucaine and 0.25 mg/ml soybean tryspin inhibitor.

The ability of pancreas mitochondria to synthesize ATP

In the present study the ability of mitochondria to synthesize ATP was also tested in addition to polarographic measurements of oxygen uptake of mitochondria to evaluate their phosphorylating capacities (Fig. 4). The 2000–6000x$g \cdot R_2$ fraction converted about 84.8% of externally added ADP into ATP. On the other hand, the 700–7000x$g \cdot R'''_2$ fraction obtained in the presence or absence of dibucaine and trypsin inhibitor converted about 97.1% and 99.2% of externally added ADP into ATP after 2 min, respectively. These data again suggest that it is not essential to add dibucaine and trypsin inhibitor to the isolation medium to obtain well-coupled mitochondria from the pancreas.

Activities of trypsin and phospholipase A_2 in pancreas mitochondria obtained by various isolation procedures

Wilson *et al.*[3] have paid special attention to avoiding the unfavorable effects of trypsin on the process of the isolation of pancreas mitochondria. Their isolation medium contained trypsin inhibitor and dibucaine to prevent trypsin and phospholipase A_2 activities. However, the present data have shown that mitochondria isolated in the absence of soybean trypsin inhibitor and dibucaine maintain a high coupling efficiency even for 8–10 hr after they are isolated. Thus, we measured trypsin and phospholipase A_2 activities in pancreas mitochondria (Table 5 & 6). Trypsin activity in the 2000–6000x$g \cdot R_2$ fraction isolated in a medium containing dibucaine and trypsin inhibitor was definitely higher than that in the 700–7000x$g \cdot R'''_2$ fraction isolated in a medium with or without dibucaine and trypsin inhibitor, and yet the activity was as low as 5 μg/mg protein in the absence of enterokinase (Table 5). When the R_2 and R'''_2 fractions were treated with enterokinase, a potent activator of trypsinogen, trypsin activities of those fractions were remarkably enhanced. These data indicate that the R_2 fraction obtained in the present study is far more contaminated with zymogen granules than the R'''_2 fraction, and yet a major portion of the enzymes remains inactivated. In the present study we have also measured phospholipase A_2 activities of pancreas mitochondria (Table 6). If trypsin activity of a mitochondrial fraction obtained in the absence of trypsin inhibitor is as low as that of a mitochondrial fraction obtained in the presence of trypsin inhibitor, phospholipase A_2 activity of the former might be as low as the latter, since phospholipase A_2 is activated by trypsin. Actually, there was no difference in phospholipase A_2 activities between the R_2 fraction isolated in the presence of trypsin inhibitor and dibucaine and that isolated in the absence of trypsin inhibitor and dibucaine. There was also no difference in the enzyme activity between the R_2 and R'''_2 fractions. Moreover, phospholipase A_2 activities in the R_2 and R'''_2 fractions isolated in the absence of EDTA, an inhibitor of phospholipase A_2 activity [18,19], remained almost unchanged for up to 8–10 hr of aging, as logn as these fractions were kept at 4°C. All these data strongly suggest that dibucaine and trypsin inhibitor could be omitted from the isolation medium to obtain intact mitochondria from the pancreas. These data suggest at the same time that trypsin activities found in these fractions might not be high enough to be injurious to mitochondrial functions. Thus, further studies were conducted to examine the effect of trypsin or dibucaine on the coupling efficiency of pancreas mitochondria. In Fig. 5A a R'''_2 fraction isolated in the absence of trypsin inhibitor and dibucaine was preincubated with trypsin (150 μg/mg protein) for 5 min at 30°C and then the rate of oxygen consumption of mitochondria was measured. The rate of oxygen consumption in state 3 respiration was unchanged while that in state 4 respiration was slightly increased. Trypsin at concentrations up to 100 μg/mg protein had no effect on mitochondrial respiration. Similar experiments were performed using rat liver mitochondria (Fig. 5B). In the case of liver mitochondria, effects of trypsin on mitochondrial respiration were not evident when its concentration was less than 100 μg/mg protein, as in the case of pancreas mitochondria, but at higher

Table 5. Trypsin activities in pancreatic mitochondria obtained by various isolation procedures[a]

Isolation Medium	Centrifugation	Fraction	Activity (μg trypsin/mg) −Enterokinase	+Enterokinase
STBE+Dib.+T.I.	2000−6000xg	R_2	5.01 ± 0.41	123.36 ± 6.30
		Homogenate	3.14 ± 0.32	205.33 ± 48.49
STBE+Dib.+T.I.	700−7000xg	R''_2	1.22 ± 0.18*	36.62 ± 4.88*
		Homogenate	3.15 ± 0.36	200.91 ± 28.50
STBE−Dib.−T.I.	700−7000xg	R''_2	1.44 ± 0.34**	40.69 ± 2.72*
		Homogenate	3.89 ± 0.08***	222.92 ± 66.96

a Isolation medium contained 0.25M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA (STBE) in the presence or absence of 400 μM dibucaine (Dib.) and 0.25 mg/ml tryspin inhibitor (T.I.). Data are the average of three different experiments (mean ± SE). Statistically different from the 200−6000x$g \cdot R_2$ fraction and the homogenate using STBE+Dib.+T.I. at; *, $p < 0.001$; **, $0.001 < p < 0.01$; ***, $0.01 < p < 0.02$.

Table 6. Phospholipase A_2 activities in pancreas mitochondria obtained by various isolation procedures[a]

Isolation Medium	Centrifugation	Fraction	Activity (nmol PE/mg/min)
STB+Dib.+T.I.+EDTA	2000−6000xg	R_2	10.7 ± 2.2
		Homogenate	15.7 ± 1.3
STB+Dib.+T.I.+EDTA	700−7000xg	R''_2	9.0 ± 2.8
		Homogenate	14.5 ± 0.2
STB−Dib.−T.I.+EDTA	700−7000xg	R''_2	10.5 ± 0.2
		Homogenate	15.6 ± 1.0
STB−Dib.−T.I.−EDTA	2000−6000xg	R_2	13.9 ± 0.3 (13.6 ± 1.4)
	700−7000xg	R''_2	12.6 ± 1.5 (13.0 ± 1.8)
		Homogenate	16.7 ± 1.8 (17.2 ± 1.6)

a Isolation medium contained 10 mM Tris-Cl, pH 7.4, 0.25M sucrose, 0.5% BSA (STB) in the presence or absence of 400 μM dibucaine (Dib.), 0.25 mg/ml soybean trypsin inhibitor (T.I.) and 1 mM EDTA. Data are the average of three different experiments (mean ± SE). Comparisons were made between the R_2 fraction obtained by a 2000−6000xg centrifugation method and the homogenate using STB+Dib.+T.I.+EDTA as the control. Data on phospholipase A_2 activities of samples aged for 10 hr at 4°C are shown in parentheses. PE: phosphatidyl ethanolamine

concentrations such as 150 μg/mg protein, the rate of oxygen consumption in state 3 respiration was decreased remarkably, with increases in state 4 respiration. On the other hand, the coupling efficiency of mitochondria treated with trypsin for 3 hr at 0°C at a concentration of 200 μg/mg protein was well maintained. These data strongly suggest that trypsin at concentrations detected in various mitochondrial fractions obtained from the pancreas in the present study would not affect the coupling efficiency of these mitochondria.

Effects of dibucaine on pancreas mitochondria were also tested. As was already demonstrated, there were not differences in the rate of state 3 and state 4 respirations and those isolated in the presence of dibucaine and those isolated in the absence of dibucaine (Table 3). However, rat liver mitochondria isolated in the presence of dibucaine at a concentration of

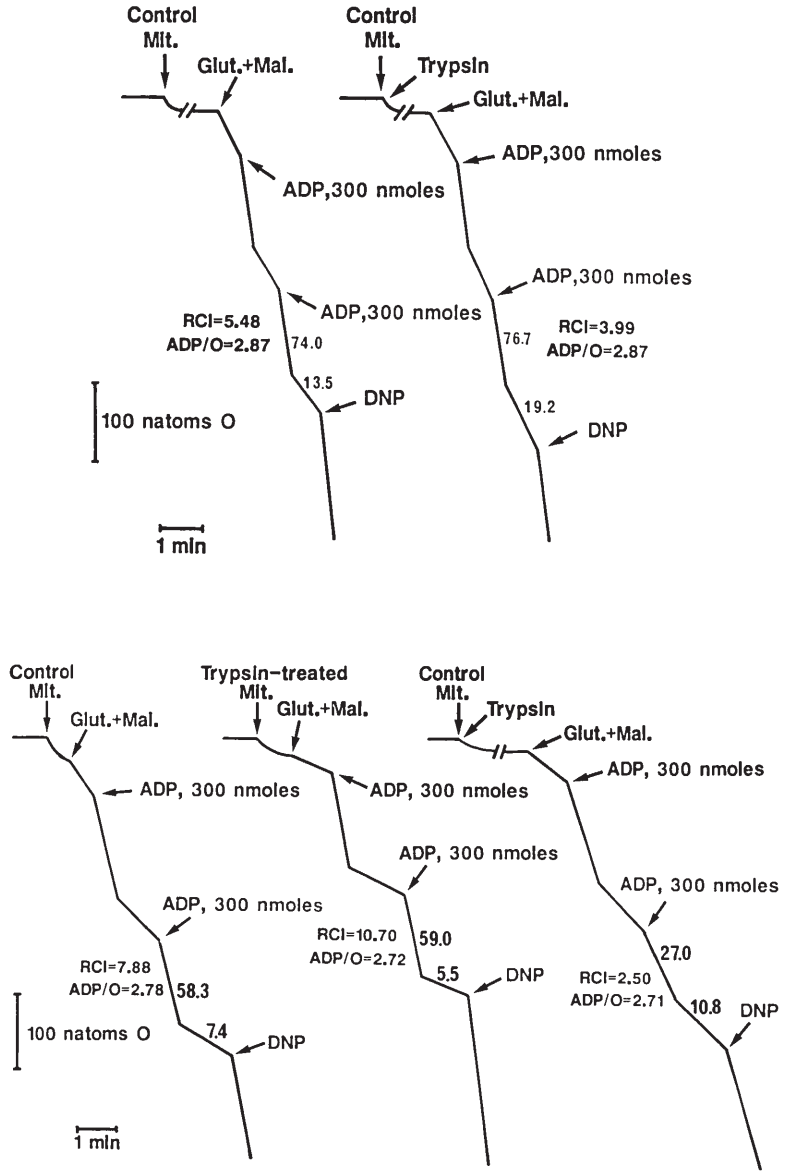


Fig. 5. Effect of externally added trypsin on mitochondrial respiration.

A. Pancreas mitochondria (a 700–7000x$g \cdot R''_2$ fraction isolated in the absence of dibucaine and trypsin inhibitor) was pretreated for 5 min at 30°C with trypsin at a concentration of 150 μg/mg protein, and then the coupling efficiency of mitochondria was measured using glutamate (+ malate) as oxidizable substrate (on the right). As the control experiment, mitochondria were preincubated for 5 min at 30°C, and then the coupling efficiency of mitochondria was measured (on the left).

B. Rate of oxygen consumption of rat liver mitochondria, pretreated for 3 hr at 0°C by trypsin at a concentration of 200 μg/mg protein, was almost unchanged (middle) compared with that of the control mitochondria (left). The rate of oxygen consumption of mitochondria in state 3 respiration pretreated with trypsin for 5 min at 30°C at a concentration of 150 μg/mg protein was decreased, while that in state 4 respiration was increased resulting in a low respiratory control index (RCI) (right).

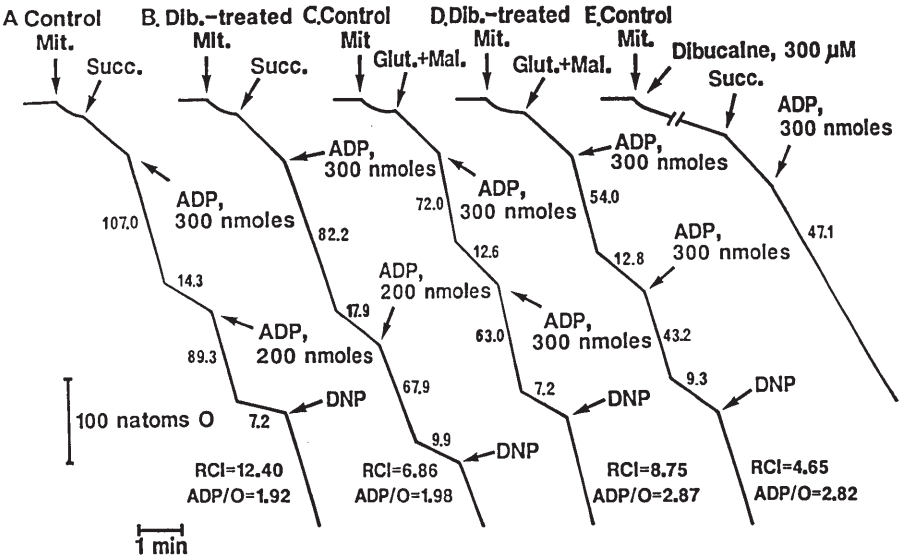


Fig. 6. Effects of dibucaine on coupling efficiency of rat liver mitochondria. The rate of oxygen consumption of mitochondria in state 3 respiration isolated in a medium containing 400 μM dibucaine was decreased when oxidizable substrate was succinate (B) or glutamate (+ malate) (D) compared with that of the control mitochondria isolated in the absence of dibucaine (A and C). Control mitochondria preincubated for 5 min at 30°C with dibucaine at a concentration of 300 μM were uncoupled (E).

400 μM (the concentration employed for the isolation of pancreas mitochondria) showed distinct decreases in the rate of state 3 respiration when either succinate or glutamate was used as substrate (Fig. 6).

DISCUSSION

There are several expected difficulties in isolating functionaly and morphologically intact mitochondria from the pancreas: (1) The pancreas acinar cells are rich in zymogens including trypsinogen and Pro-phospholipase A_2 which may be activated and damage mitochondria when they are being isolated. (2) The organ is heterogenous in structure. Acinar cells are grouped into lobules by fibrous connective tissues. This causes difficulty in the homogenization. If the tissue is homogenized too much, mitochondria may be disrupted, while insufficient homogenization of the tissue may yield a smaller amount of mitochondria. The organ is not only covered with a large amount of adipose tissue but fatty infiltration is inevitably found within intra- and interlobular spaces. Unsaturated fatty acids are known to swell and uncouple mitochondria. All these have made it difficult for us to establish an isolation procedure for intact mitochondria from the pancreas. In the literature, Wilson *et al.*[3] are the first to report success in isolating intact mitochondria from the pancreas. Their isolation medium contained soybean trypsin inhibitor since trypsin is the trigger enzyme responsible for activating all other pancreatic zymogens[19]. It also contained dibucaine and 1 mM EDTA to inhibit phospholipase A_2 which might attack membrane phospholipids. A high concentration of BSA (0.5%) was also added to their isolation medium. However, they did not present data on the question of whether or not trypsin inhibitor and dibucaine were absolutely necessary to obtain functional mitochondria. Two ways of

activating trypsinogen to trypsin are known: autoactivation and activation by enterokinase present in the duodenal juice[19]. Once the pancreas is removed from the animal, there is no chance for tripsinogen to be activated by enterokinase. Thus, the effects of trypsin produced by the autoactivation should be taken into account when mitochondria are isolated from the pancreas. If the rate constant for enterokinase-catalyzed trypsinogen activation exceeds that for the autoactivation by several orders of magnitude[19], it is possible that isolated mitochondria are not damaged by trypsin. The present study has confirmed that the procedure developed by Wilson *et al.*[3] is useful in detecting phosphorylating capacities of pancreas mitochondria especially when a small amount of tissue is available. However, when it is necessary to look into the details of the functional aspects of pancreas mitochondria, such as changes in membrane fluidity, anion transport, permeability to various drugs, activities of various enzymes, it would be better to avoid the use of dibucaine and trypsin inhibitor in the isolation medium for mitochondria because evidence indicates anesthetics modify the structure and function of biological membrane systems including mitochondria[20–28]. The present study has shown that intact pancreas mitochondria can be obtained without using either a special isolation medium or a special centrifugation method.

Yield of mitochondria with respect to the wet weight of the pancreas or the homogenate

The present study demonstrated that the yield of mitochondria on the protein basis relative to the homogenate of the pancreas was extremely low compared with that of the liver although the ratio of the homogenate to the wet weight of the tissue was even higher in the former. Meldolesi *et al.*[29] also obtained a low yield of mitochondria from the guinea pig pancreas although their isolation procedure for mitochondria was different from that tested in the present study (mitochondria/wet weight of the pancreas, 0.2%, mitochondria/homogenate, 1.39%). Since the ratio of homogenate to the wet weight of the tissue was higher in the pancreas than in the liver, and the content of proteins per wet weight of the tissue is larger in the former[30,31], a smaller contribution of mitochondrial proteins to the total amount of tissue proteins in the pancreas may be the major reason for obtaining a low yield of pancreas mitochondria. Actually, the number of mitochondria per acinar cell revealed on a plane section is definitely smaller than that of the hepatocyte when judged on electron micrographs (unpublished obserbvation). Another possible reason may be that some of the mitochondria were broken and lost during the homogenization because of the structural heterogeneity described above, despite special care taken when the pancreas was homogenized. If we focus only on the yeild of mitochondria relative to 1 g of the wet weight of the tissue, a total of 3–4 mg for the R''_2 fraction and 11–15 mg for the R_2 fraction were obtained. When the R_2 fraction is used as a mitochondrial fraction in case, for example, individual rat pancreas is used as a starting material, a 2000–6000x*g* centrifugation method is recommended rather than a 700–7000x*g* centrifugation method, since the contamination of the latter with other organelles is severer when judged by the content of cytochromes. When enough of the tissue is available as a starting material a R''_2 fraction using a 700–7000x*g* centrifugation method is recommended rather than that using a 2000–6000x*g* centrifugation method based on cytochrome contents, especially of $a+a_3$ and b (Table 2), and activities of cytochrome oxidase and ATPase (Table 4) indicating that the former is less contaminated with other organelles than the latter.

Correlation between coupling efficiency of pancreas mitochondria and the composition of isolation medium

We have shown in the present study that the coupling efficiency of pancreas mitochondria is well preserved as long as the isolation medium contains both BSA and EDTA. Rates of oxygen

consumption in state 3 and state 4 respirations of the 2000–6000x*g* R_2 fraction isolated in the presence of dibucaine and trypsin inhibitor were definitely low compared with those of the 2000–6000x*g* · R'''_2 or the 700–7000x*g* · R'''_2 fraction isolated in the presence or absence of dibucaine and trypsin inhibitor (Table 3). However, respiratory control ratios of the former were as high as the latter. These data suggest that the lower rates of respiration of the former fraction were due to the contamination with other organelles and that neither dibucaine nor trypsin inhibitor was essential to obtain well-coupled mitochondria. Namely, trypsinogen was not activated during the homogenization and the separation of the mitochondrial fraction. Sardesai and Provido[32] also could not detect trypsin activity in their mitochondrial fraction or in the homogenate of the tissue. To summarize, the present results suggest that the presence of dibucaine and trypsin inhibitor is not an absolute requirement for the isolation of functional mitochondria from the pancreas. Finally, we should refer briefly to the cytochrome content of pancreas mitochondria. As already shown in the previous section, we have obtained the highest concentration of cytochromes in the 700–7000x*g* · R'''_2 fraction (Table 2). In the present study a differential centrifugation method was applied to the pancreas and further attempts were not made to purify mitochondria. The averaged concentration of cytochromes obtained in pancreas mitochondria suggests that they are still contaminated with other organelles to some extent since pancreas mitochondria are almost as rich in the inner membranes as heart mitochondria. Among the data on cytochrome content of pancreas mitochondria obtained in the presnet study, the highest values obtained in one experiment were: $a+a_3$, 0.365; *b*, 0.296, c_1, 0.148 and *c*, 0.101 ($c+c_1$, 0.249). In another experiment, a R_2 fraction obtained by a 700–7000xg centrifugation method was washed 4 times, and the content of cytochromes in the fraction was $a+a_3$, 0.371; *b*, 0.351; c_1, 0.136, and *c*, 0.131 ($c+c_1$, 0.267). Thus, a higher content of cytochromes would be obtained from pancreas mitochondria if the isolation procedure were improved further.

ACKNOWLEDGEMENTS

We are grateful to Dr. K. Tamiya-Koizumi, Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, for the estimation of phospholipase A_2 activity. We thank Miss K. Hasegawa for the preparation of the manuscript. This work was supported in part by a grant for Intractable Pancreatic Disease from the Ministry of Welfare of Japan.

REFERENCES

1) Leiber, C.S.: Pathogenesis of alcoholic fatty liver. In *Medical disorders of alcoholism. Pathogenesis and treatment*, edited by Lieber, C.S., pp. 146–159 (1982), W.B. Saunders Company, Philadelphia.
2) Noronha, M., Salgadhino, A., Ferreira de Almedia, M.J., Dreiling, D.A. and Bordalo, O.: Alcohol and the pancreas. I. Clinical associations and histopathology of minimal pancreatic inflammation. *Am. J. Gastroenterol.*, 76, 114–119 (1981).
3) Wilson, J.S., Korsten, M.A., Leo, M.A. and Lieber, C.S.: New technique for the isolation of functional rat pancreatic mitochondria and its application to models of pancreatic injury. *J. Lab. Clin. Med.*, 107, 51–58 (1986).
4) Kishino, Y., Takama, S. and Kitajima, S.: Ultracytochemistry of pancreatic damage induced by excess lysine. *Virchows Arch. Cell Pathol.*, 52, 153–167 (1986).
5) Sardesai, V.M. and Provido, H.S.: The presence of a substance in the pancreas that inhibits mitochondrial respiration. *J. Surg. Res.*, 35, 176–179 (1983).
6) Lowry, O.H., Rosebrough, N.J., Farr, L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265–275 (1951).

7) Estabrook, R.W.: Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. *Methods Enzymol.*, 10, 41–47 (1967).
8) Wakabayashi, T., Asano, M. and Kawamoto, S.: Induction of megamitochondria in the mouse liver by isonicotinic acid derivatives. *Exp. Mol. Pathol.*, 31, 387–399 (1979).
9) Wakabayashi, T., Horiuchi, M., Sakaguchi, M., Onda, H. and Misawa, K.: Induction of megamitochondria in the mouse and rat livers by hydrazine. *Exp. Mol. Pathol.*, 39, 139–153 (1983).
10) Fiske, C.H. and Subbarow, Y.: The colorimetric determination of phosphorus. *J. Biol. Chem.*, 66, 375–400 (1925).
11) Hayakawa, T., Kondo, T., Yamazaki, Y., Iinuma, Y. and Mizuno, R.: A simple and specific determination of trypsin in human duodenal juice. *Gastroenterologia Japonica*, 15, 135–139 (1980).
12) Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., Ishihara, H. and Kojima, K.: A novel phospholipase A_2 associated with nuclear matrix: stimulation of the activity and modulation of the Ca^{2+} dependency by polyphosphoinositides. *Biochim. Biophys. Acta.*, 1002, 182–188 (1989).
13) McElory, W.D. and DeLuca, M.: Bioluminescence and chemiluminescence. Basic chemistry an analytical applications. In *The chemistry and analytical applications of firefly luminescence.* edited by McElroy, W.D. and DeLuca, M.A., pp. 179–186 (1981), Academic Press, New York.
14) Takagi, S., Mizuno, N., Adachi, K. and Wakabayashi, T.: Effects of demethylchlortetracycline phototoxicity on the structure and functions of rat liver mitochondria. *Photochem. Photobiol.*, 51, 175–183 (1990).
15) Williams, J.N., Jr.: A method for the simultaneous quantitative estimation of cytochromes *a*, *b*, c_1 and *c* in mitochondria. *Arch. Biochem. Biophys.*, 107, 537–543 (1964).
16) Wakabayashi, T., Horiuchi, M., Sakaguchi, M., Misawa, K., Onda, H., Iijima, M. and Allmann, D.W.: Mechanism of hepatic megamitochondrial formation by ammonia derivatives. Correlation between structure of chemicals and their ability to induce the formation of megamitochondria. *Eur. J. Biochem.*, 143, 455–465 (1984).
17) Wakabayashi, T. and Green, D.E.: On the mechanism of cuprizone-induced formation of megamitochondria in mouse liver. *Bioenergetics*, 6, 179–192 (1974).
18) Tykka, H., Mahlberg, K., Pantzar, P. and Tallberg, T.: Phospholipase A_2 inhibitors and their possible clinical use in the treatment of acute pancreatitis. *Scand. J. Gastroent.*, 15, 519–528 (1980).
19) Rinderknecht, H.: Pancreatic secretory enzymes. In *The exocrine pancreas: biology, pathology, and diseases.* edited by Go, V.L.W., Books, F.P., DiMagno, E.P., Gardner, J.D., Lebenthal, E. and Scheele, G.A., pp. 163–183 (1986), Ravan Press, New York.
20) Miller, R.N. and Hunter E.F.JR.: The effect of halothane on electron transport-oxidative phosphorylation and swelling in rat liver mitochondria. *Mol. Pharmacol.*, 6, 67–77 (1970).
21) Harris, R.A., Munroe, J., Farmer, B., Kim, K.C. and Jenkins, P.: Action of halothane upon mitochondrial respiration. *Arch. Biochem. Biophys.*, 142, 435–444 (1971).
22) Jain, M.K., Wu, N.Y. and Wray, L.V.: Drug-induced phase change in bilayer as possible mode of action of membrane expanding drugs. *Nature*, 225, 494–496 (1975).
23) Tsong, T.Y., Greenberg, M. and Kanehisa, M.I.: Anesthetic action on membrane lipids. *Biochemistry*, 16, 3115–3121 (1977).
24) Vanderkooi, J.M., Landesberg, R., Selick. H. and McDonald, G.C.: Interaction of general anesthetics with phospholipid vesicles and biological membranes. *Biochim. Biophys. Acta.*, 464, 1–16 (1977).
25) Trudell, JR.: Biophysical concept in molecular mechanism of anesthesia. In *Molecular Mechanism of Anesthesia. Progress in Anesthesiology vol.2.* edited by Fink, B.R., pp. 267–270 (1980) Raven Press, New York.
26) Vanderkooi, G., Shaw, J., Storm, S.C., Vennerstrom, R. and Chignell, D.: On the mechanism of action of anesthetics. Direct inhibition of mitochondrial F-ATPase by n-butanol and tetracaine. *Biochim. Biophys. Acta*, 635, 200–203 (1981).
27) Dluzewski, A.R., Halsey, M.J. and Simmonds, A.C.: Membrane interactions with general and local anesthetics: A review of molecular hypothesis of anesthesia. *Molec. Aspects Med.*, 6, 459–573 (1983).
28) Ueda, I. and Kawaya, H.: Molecular mechanisms of anesthesia. *Anesth. Analg.*, 63, 929–945 (1984).
29) Meldolesi, J., Jamieson, J.D. and Palade, G.E.: Composition of the cellular membranes in the pancreas of the guinea pig. I. Isolation of membrane fractions. *J. Cell Biol.*, 40, 109–129 (1971).
30) Black, D.A.K. and Lumb, G.A.: Chemical composition of the alimentary tract. In *Biochemists' Handbook.* edited by Long, C., pp. 675–676 (1961) Richard Clay and Company, Suffolk, England.
31) Kosterlitz, H.W.: Chemical composition of the liver. In *Biochemists' Handbook.* edited by Long, C., pp. 675–676 (1961) Richard Clay and Company, Suffolk, England.
32) Sardesai, V.M. and Provido, H.S.: The presence of a substance in the pancreas that inhibits mitochondrial respiration. *J. Surgical. Res.*, 35, 176–179 (1983).