

# Loose-coupled mitochondria in chronic glucagon-treated hyperthermic ducklings

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BARRÉ, HERVÉ, GILLES BERNE, PIERRE BREBION, FRÉDÉRIQUE COHEN-ADAD, AND JEAN LOUIS ROUANET. *Loose-coupled mitochondria in chronic glucagon-treated hyperthermic ducklings*. Am. J. Physiol. 256 (Regulatory Integrative Comp. Physiol. 25): R1192-R1199, 1989.—In chronic glucagon-treated ducklings (GT) showing thermogenic and hyperthermic responses without shivering to glucagon test injection and in control ducklings (TN; both aged  $44 \pm 1$  days and reared at thermoneutrality), subsarcolemmal (S) and intermyofibrillar (I) mitochondria from gastrocnemius muscle and mitochondria from liver were isolated. Respiration and cytochrome oxidase activity were determined in these isolated mitochondria by polarography and creatine kinase activity by spectrophotometry, both at 25°C. In GT ducklings, the powerful thermogenesis observed in vivo after a glucagon test injection may be due to the uncoupling effect of released free fatty acids (FFA) in loose-coupled mitochondria because their respiration increased as a function of FFA concentration, and the loose coupling of these mitochondria was reversed by addition of albumin. In all types of mitochondria from GT ducklings, the increase in respiration because of FFA was about double that in mitochondria from controls. There was no change in creatine kinase activity from liver and I mitochondria, but a 16% decrease in this enzyme activity (expressed per mg mitochondrial protein) from S mitochondria was shown despite a strong increase in cytochrome oxidase activity from liver mitochondria (+114% if expressed per g tissue) and from muscle mitochondria (I, +53 or +48%; S, +41 or +97% if expressed per mg mitochondrial protein or per g tissue, respectively). These results support a coupling defect in liver and skeletal muscle mitochondria from the GT hyperthermic ducklings and an uncoupling reinforcement by FFA.

birds; nonshivering thermogenesis; acclimation to cold; mitochondrial respiration; cytochrome oxidase activity; creatine kinase activity; uncoupling effect of free fatty acids

IN YOUNG BIRDS, cold acclimation can induce the development of nonshivering thermogenesis (NST; 5, 7). Likewise, chronic glucagon treatment of ducklings reared at thermoneutrality induces NST in the cold (6). In mammals, NST triggered by norepinephrine is based on increased respiration of brown adipose tissue (BAT) in which mitochondria are uncoupled (27). In consideration of the lack of thermogenic BAT in birds (5, 22), the liver and skeletal muscle would be possible sites of such a thermogenesis. Indeed, in mammals, loose-coupled muscle mitochondria have already been found in cold-accli-

mated white mice (35) and in cold-acclimatized fur seal pups (18).

In mammals, free fatty acids (FFA) released by lipolysis caused by norepinephrine are shown to be responsible for uncoupling in BAT mitochondria from cold-acclimated hamsters and guinea pigs (24, 25, 31). Similarly, in a bird devoid of BAT (the cold-acclimated duckling) (5), FFA led by uncoupling to increased respiration in skeletal muscle mitochondria (9). The NST observed in cold-acclimated or glucagon-treated (GT) ducklings could be attributed to the same uncoupling effect of FFA released by secreted or injected glucagon. Such a possible mechanism of regulation was looked for in vitro to evaluate the ability of released FFA to uncouple muscle and/or liver mitochondria from chronically GT ducklings.

Another sign of involvement of a tissue in a metabolic process is the enzyme activity. Cytochrome oxidase activity is generally used as an index of the aerobic oxidative capacity of mitochondria or organs (21). In muscle, the creatine phosphate shuttle appears to be an essential step for energy release (12, 20), and the mitochondrial creatine kinase activity, the adaptive character of which has been demonstrated (2, 10, 34), may be considered an excellent index of phosphorylative activity of this tissue. An increased oxidative capacity of skeletal muscles (3, 4, 9) and liver (3, 4) has been shown in cold-acclimated birds, but as yet no modification of phosphorylative activity has been looked for simultaneously. Indeed a discrepancy between the activity of both enzymes before and after the chronic treatment (increase in oxidative capacity without corresponding modification of phosphorylative capacity or with a decrease in this latter capacity) could indicate a mitochondrial coupling defect. To disclose the involvement of muscle or liver in the adaptive increase in thermogenic response from GT ducklings, the activity of both these enzymes engaged in the metabolic process of energy expenditure was measured in mitochondria from these organs.

## MATERIALS AND METHODS

*Animals.* Twenty-four male muscovy ducklings (*Cairina moschata*, L., pedigree R31, Institut National de la Recherche Agronomique, France) from a commercial stockbreeder (Ets Grimaud) were used. These were fed a commercial mash (Sanders 5061) ad libitum and had free access to water. The glucagon treatment schedule de-

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scribed by Barré et al. (6) was used. From the age of 1 wk, the ducklings were caged for a period of 6–7 wk at 25°C ambient temperature and were treated twice daily with glucagon (360 µg/kg ip; GT) or saline solution [controls (TN)]. They were kept in a constant photoperiod (a light-dark cycle, 8:16 h). At the age of 44 ± 1 days, the GT and TN ducklings, weighing 1,970 ± 84 and 2,474 ± 65 g, respectively, were killed by decapitation to examine the functional properties of liver and muscle mitochondria in vitro.

**Skeletal muscle mitochondria.** Muscle subsarcolemmal and intermyofibrillar mitochondria were isolated from the gastrocnemius muscle by a modification of the procedure of Palmer et al. (28) and of Wardlaw and Kaplan (36) for rat muscle mitochondria. The gastrocnemius muscles were quickly removed and placed in ice-cold isolation *buffer A* (see below for composition). They were then trimmed of fat and connective tissue, blotted dry, weighed (5 g in both GT and TN groups), and finally minced with scissors. The mince, suspended in 25 ml isolation *buffer A*, was homogenized with a Potter-Elvehjem homogenizer (5 passages). The homogenate was centrifuged at 800 g for 10 min. The pellet containing most of the intermyofibrillar mitochondria (because they are associated with the myofibrils; Fig. 1) was suspended in 40 ml isolation *buffer A* and kept on ice during the isolation of the subsarcolemmal mitochondria.

The supernatant containing the subsarcolemmal mitochondria was centrifuged at 1,000 g for 10 min and then at 8,700 g for 10 min. The resulting pellet was suspended in 20 ml of isolation *buffer A* and recentrifuged at 8,700 g for 10 min. The resulting pellet resuspended in 10 ml *buffer B* was then washed by centrifugation at 8,700 g for 10 min. The final subsarcolemmal mitochondrial pellet (not shown) was suspended in a minimal volume of *buffer B* and kept on ice.

Nagarse (1 mg/g muscle wet wt) was added to the intermyofibrillar mitochondrial homogenate and incubated for 5 min in an ice bath. The mixture was diluted with 40 ml of the same *buffer A* (without nagarse), homogenized, and centrifuged at 1,000 g for 10 min to pelletize the myofibrils. The resulting supernatant containing the intermyofibrillar mitochondria was filtered through cheese cloth and centrifuged at 8,700 g for 10 min. The resulting pellet was suspended in 20 ml *buffer A* and recentrifuged at 8,700 g for 10 min. The resulting pellet was suspended in 10 ml *buffer B* and washed by centrifugation at 8,700 g for 10 min. The final intermyofibrillar mitochondrial pellet (not shown) was resuspended in a minimal volume of storage *buffer B* and kept on ice. All procedures were carried out at 4°C.

After the determination of mitochondrial protein by the biuret method (with bovine serum albumin as standard), the mitochondria were diluted to 20 mg/ml in *buffer B*. For the subsarcolemmal and intermyofibrillar mitochondria, the yield was 8.2 ± 0.5 and 32.9 ± 3.3 mg protein/preparation in TN ducklings and 11.0 ± 0.8 and 35.0 ± 2.9 mg protein/preparation in GT ducklings, respectively.

**Isolation and storage medium for muscle mitochondria.** The muscle was homogenized in an isolation medium

(*buffer A*) consisting of (in mM) 100 sucrose, 50 tris(hydroxymethyl)aminomethane (Tris) base (adjusted pH 7.4), 5 MgCl<sub>2</sub>, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 100 KCl, and 1 ATP. The isolated muscle mitochondria were kept in a storage medium (*buffer B*) containing (in mM) 250 sucrose, 20 Tris base (adjusted pH 7.4), and 1 EGTA.

**Liver mitochondria.** Liver mitochondria were prepared by the procedure of Nedergaard and Cannon (26). Briefly, liver was homogenized with a Potter-Elvehjem homogenizer in an isolation medium consisting of (in mM) 250 sucrose, 20 Tris·HCl (adjusted pH 7.0), and 0.1 EGTA. The homogenate was centrifuged at 800 g for 10 min. The supernatant containing the liver mitochondria was centrifuged at 1,000 g for 10 min and then at 8,700 g for 10 min. The pellet was resuspended in the same medium and recentrifuged as before. The final liver mitochondrial pellet (not shown) was resuspended in a minimal volume of the medium and kept on ice. Liver mitochondria were then treated as muscle mitochondria. The yield was 15.5 ± 1.2 and 25.9 ± 1.9 mg mitochondrial protein/preparation in TN and GT ducklings, respectively.

**Mitochondrial respiration.** The respiration of isolated mitochondria (0.5 mg mitochondrial protein/ml) was determined polarographically (15) at 25°C, using a Clark O<sub>2</sub> electrode, in 1 ml of a respiratory medium consisting of (in mM) 200 sucrose (for muscle mitochondria) or 125 sucrose (for liver mitochondria), 4 Tris-PO<sub>4</sub>, 20 Tris·HCl (adjusted pH 7.2), 2 MgCl<sub>2</sub>, 5 Na succinate, and 5 µM rotenone. State III respiration was initiated by the addition of 100 µM ADP, and the method of Estabrook (15) was used for the calculation of states IV and III respiration and respiratory control ratio (RCR). The sensitivity to the uncoupling effect of FFA was measured, as previously described (9), by the addition of 10 mM glucose, 10 U hexokinase, 0.5 mM guanosine 5'-diphosphate (GDP), and 1 µg/ml oligomycin but without albumin in the medium because mitochondria were isolated and stored in the absence of fatty acid-free bovine serum albumin (FAF-BSA).

**Cytochrome oxidase activity.** Cytochrome oxidase activity in muscle and liver mitochondria was determined polarographically at 25°C, using a Clark O<sub>2</sub> electrode and a modified procedure (4) of Aulie and Grav (3), in 1 ml of reaction medium containing 30 µM cytochrome c, 4 µM rotenone, 0.5 mM dinitrophenol (DNP), 10 mM N-malonnate, and 75 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (adjusted pH 7.4). Muscle and liver mitochondria diluted in a modified Chappell-Perry medium [(in mM) 1 ATP, 50 HEPES buffer (adjusted pH 7.4), 100 KCl, 5 MgCl<sub>2</sub>, 1 EDTA, and 5 EGTA] corresponded to 0.1 mg mitochondrial protein. Lubrol (100 mg/g mitochondrial protein) was used to unmask enzyme activity in mitochondria that were standing in ice for 30 min. Cytochrome oxidase activity was measured as the difference between the rate of O<sub>2</sub> consumption observed after the addition of substrate (4 mM Na-ascorbate with 0.3 mM TMPD) and mitochondria and the rate of O<sub>2</sub> consumption observed after the addition of substrate alone to take the autoxi-

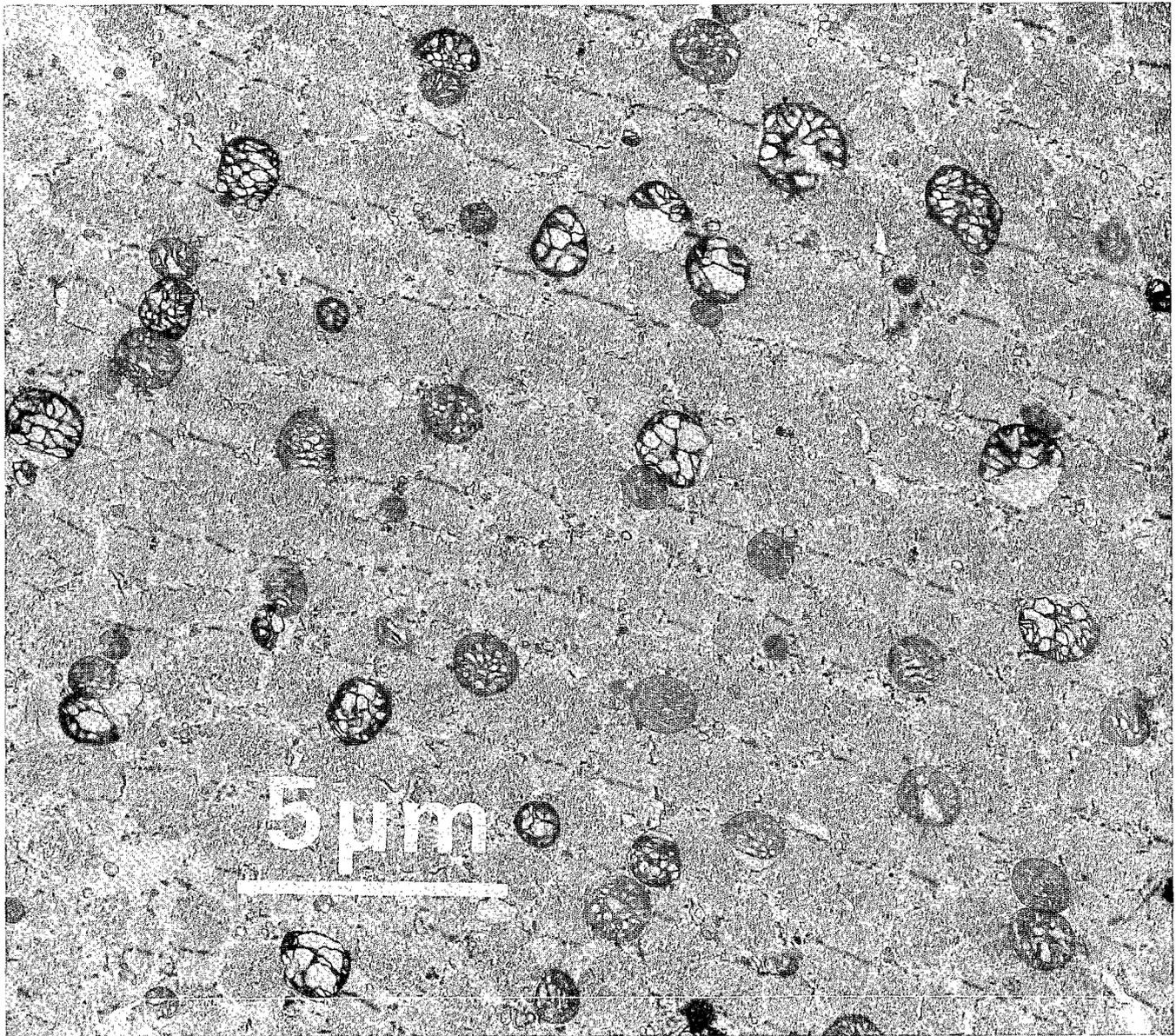


FIG. 1. Electron micrograph of intermyofibrillar mitochondria within myofibrils before treatment with nagarse.

dation of ascorbate into account.

**Creatine kinase activity.** Creatine kinase activity was measured by spectrophotometry at 25°C (pH 9.0) by a modified procedure of Foster et al. (16) and using creatine as substrate in 1.040 ml reaction medium containing coenzyme-glycine buffer [(in mM) 0.75  $\beta$ -NADH, 6.1 ATP, 1.5 phosphoenolpyruvate (PEP), 6.1  $MgCl_2$ , and 675 glycine (pH 9.0)], creatine-glycine buffer [(in mM) 35.6 creatine in 56 glycine buffer (pH 9.0)], 9.0 mM glutathione, and lactate dehydrogenase-pyruvate kinase (each 80  $\mu$ g). Mitochondria diluted in *buffer B* for muscle were used at 1 mg mitochondrial protein/ml (for subsarcolemmal and intermyofibrillar mitochondria) or at 4 mg mitochondrial protein/ml (for liver mitochondria). Lubrol (100 mg/g mitochondrial protein) was used to unmask enzyme activity in mitochondria that were standing in ice for 30 min. Creatine kinase activity was measured at 340 nm against a blank containing all the solutions

with glycine buffer (0.1 M, pH 9.0) instead of creatine-glycine buffer.

**Control experiments.** The nagarse action on respiration and enzyme activity was measured on isolated liver and subsarcolemmal mitochondria (extracted without the use of this proteolytic enzyme). No significant differences ( $P > 0.05$ ) were found with or without the use of nagarse (to a concentration of 0.4 or 0.8 mg/mg mitochondrial protein) in both types of mitochondria. These results confirmed the isolation of two populations of mitochondria from muscle.

**Electron microscopy.** To ensure the purity of the isolated mitochondria, each final mitochondrial pellet of muscle and liver was checked by electron microscopy. The centrifugation pellets were carefully removed from the tube and quickly placed in ice-cold fixative (0.5% paraformaldehyde and 2% glutaraldehyde in 100 mM Na-K phosphate buffer, pH 7.4) for 3 h. The pellets were

then washed in ice-cold 175 mM Na-K phosphate buffer (pH 7.4) for 2 h at 4°C and postfixed (2% osmium tetroxide in 100 mM Na-K phosphate buffer, pH 7.4) for 2 h at room temperature. The fixed pellets were quickly dehydrated in ice-cold ethanol and embedded in epon as described by Barré et al. (5). Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and studied under a Hitachi HU-12-A electron microscope in the microscopy center of the University of Lyon.

**Materials.** Palmitic acid (sodium salt diluted in ethanol), Tris, DNP, Na malonate, rotenone, oligomycin, and nagarse were purchased from Sigma Chemical (St. Louis, MO). FAF-BSA, hexokinase, pyruvate kinase, lactate dehydrogenase, ATP, ADP, GDP, NADH, PEP, glutathione, HEPES, and cytochrome *c* were from Boehringer Mannheim (Mannheim, FRG). Glycine, creatine, EDTA, and EGTA were from Prolabo (Paris, France); lubrol was from Serva (Heidelberg, FRG); sucrose was from Merck (Darmstadt, FRG); and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was from Fluka (Buch, Switzerland). Other chemicals were pro analysi grade.

**Units and statistics.** International units have been used throughout this paper. Values have been presented as means  $\pm$  SE. Student's *t* test, analysis of variance (ANOVA), and appropriate post hoc tests were used for statistical calculations.

## RESULTS

In the 44-day-old GT ducklings compared with the controls, chronic glucagon treatment resulted (Table 1) in a significantly lower body mass but a higher liver mass. The mass of liver mitochondria and subsarcolemmal mitochondria from gastrocnemius muscle was also increased in GT ducklings, whereas the mass of intermyofibrillar mitochondria from gastrocnemius muscle did not change.

**Effect of glucagon treatment and FFA on mitochondrial respiration (Table 2).** Respiration of the intermyofibrillar and subsarcolemmal mitochondria from gastrocnemius muscle and of mitochondria from liver isolated in the absence of FAF-BSA was measured with succinate as substrate. To allow only oxidation of this flavoprotein-coupled substrate, the respiratory medium contained rotenone, which inhibits NADH-linked respiration (i.e., fatty acid oxidation). Data for state IV, state III, RCR values, and the uncoupling effect of FCCP were analyzed

by two-way ANOVA, using chronic glucagon treatment and the uncoupling effect of FFA as experimental treatments. This analysis indicated a significant effect of the chronic glucagon treatment on the RCR values that were decreased, showing loose-coupled mitochondria from GT ducklings, a significant effect of FFA, and no interaction between the two treatments. When the three types of isolated mitochondria were incubated in the presence of FFA, state IV respiration was significantly increased, whereas state III respiration was not significantly different from state III respiration in the absence of FFA. Consequently, the RCR of these mitochondria decreased in both GT and TN ducklings, showing an uncoupling effect of FFA. This uncoupling effect of FFA was observed from 0.5 and 1.5 nmol/ml in the intermyofibrillar mitochondria of GT and TN ducklings, respectively, and from 0.5 and 1.5 nmol/ml in the liver and subsarcolemmal mitochondria of both GT and TN ducklings, respectively.

**Effect of FAF-BSA on the mitochondrial coupling state (Table 2).** Muscle and liver mitochondria isolated in the absence of BSA were better coupled after an addition of FAF-BSA (0.15%) to the respiratory medium. In particular, it is possible to reverse the loose coupling of mitochondria from GT ducklings, and RCR values become not significantly different from that of controls.

**Sensitivity to the uncoupling effect of FFA (Table 3).** To measure specifically this uncoupling effect and not the effects of acyl-CoA derivatives of the fatty acids, the respiratory medium also contained an ATP trap in the form of hexokinase plus glucose, together with the ATP synthesis inhibitor oligomycin. Before the addition of FFA, the basal respiration (measured during 3 min) of the three types of mitochondria was not significantly different between GT and TN ducklings. The respiration caused by the uncoupling effect of FFA (measured during 3 min) was significantly increased by the addition of 1.5 nmol/ml FFA in intermyofibrillar mitochondria ( $F_{GT} = 54.4$ ,  $P < 0.001$ ;  $F_{TN} = 19.1$ ,  $P < 0.05$ ), 5 nmol/ml FFA in subsarcolemmal mitochondria ( $F_{GT} = 31.1$ ,  $P < 0.001$ ;  $F_{TN} = 13.8$ ,  $P < 0.01$ ) from both GT and TN ducklings, and 10 nmol/ml FFA in liver mitochondria ( $F_{GT} = 9.2$ ,  $P < 0.05$ ) from GT ducklings. The increase in respiration caused by the specific uncoupling effect of FFA (Fig. 2) was about twice as high in mitochondria from GT ducklings as in mitochondria from TN ducklings. The stimulation of respiration occurred within a small concentration range of FFA, particularly in intermyofibrillar mi-

TABLE 1. Effect of glucagon treatment on body and liver mass and on yield of mitochondrial protein of gastrocnemius muscle and liver from ducklings

	Control	Glucagon Treated	Variation, %
Body mass, g	2,474.1 $\pm$ 64.2	1,969.7 $\pm$ 84.4‡	-20.4
Liver mass, g	65.0 $\pm$ 2.5	81.2 $\pm$ 4.8*	+24.9
Liver mass/body mass, %	2.64 $\pm$ 0.13	4.11 $\pm$ 0.14	
Mitochondrial protein mass, mg/g <sub>w</sub>			
Gastrocnemius muscle			
Subsarcolemmal mitochondria	1.61 $\pm$ 0.10	2.22 $\pm$ 0.17†	+37.9
Intermyofibrillar mitochondria	6.61 $\pm$ 0.66	7.07 $\pm$ 0.64	
Liver mitochondria	7.75 $\pm$ 0.72	13.23 $\pm$ 1.00†	+70.1

Values are means  $\pm$  SE for 6 ducklings in each group. \*  $P < 0.05$ , †  $P < 0.01$ , and ‡  $P < 0.001$ .

TABLE 2. *Respiration of gastrocnemius muscle mitochondria and liver mitochondria from control and glucagon-treated ducklings*

FFA, nmol/ml: FAF-BSA %:	Respiration, nmol O · min <sup>-1</sup> · mg protein <sup>-1</sup>							
	TN				GT			
	0	0	0.5	1.5	0	0	0.5	1.5
Gastrocnemius muscle								
Intermyofibrillar mitochondria								
State IV	42.9±4.3	39.9±3.7	48.8±4.7	57.3±4.9	36.1±3.0	29.7±5.2 <sup>d</sup>	49.7±8.7	57.8±5.4 <sup>e</sup>
State III	200.8±18.1	261.6±23.5 <sup>d</sup>	175.3±16.5	167.1±15.9	125.8±13.4	167.7±29.0	122.0±13.1	113.0±20.5
RCR	4.12±0.25	6.59±0.28 <sup>e</sup>	3.59±0.06	3.17±0.18 <sup>c</sup>	3.48±0.07 <sup>a</sup>	5.80±0.39 <sup>e</sup>	2.55±0.22 <sup>ab</sup>	1.92±0.25 <sup>ac</sup>
FCCP	202.5±19.6	306.7±27.5 <sup>e</sup>	166.1±18.8	162.8±17.8	136.0±13.0	191.1±27.8 <sup>d</sup>	124.9±12.6	107.6±23.8
Subsarcolemmal mitochondria								
State IV	27.7±2.8	19.6±1.4	30.2±2.1	33.4±1.6 <sup>e</sup>	25.4±1.5	17.1±1.1 <sup>d</sup>	33.0±1.6 <sup>b</sup>	34.6±2.8 <sup>b</sup>
State III	59.2±5.5	77.1±9.5	56.7±4.4	55.7±3.2	45.7±3.1	54.3±3.3	46.5±4.5	47.4±5.2
RCR	2.14±0.18	3.96±0.52 <sup>d</sup>	1.89±0.14	1.68±0.12 <sup>b</sup>	1.82±0.16 <sup>a</sup>	3.24±0.33 <sup>d</sup>	1.42±0.13 <sup>a</sup>	1.37±0.13 <sup>ab</sup>
FCCP	59.2±5.1	70.9±6.6	56.5±5.0	55.2±3.6	51.0±4.9	61.6±6.2 <sup>d</sup>	50.8±4.7	50.4±5.6
Liver								
Liver mitochondria								
State IV	14.9±1.5	9.5±0.8	18.2±1.5	26.1±2.4 <sup>e</sup>	10.9±1.5	6.0±0.4 <sup>d</sup>	20.3±1.2 <sup>b</sup>	24.0±2.6 <sup>b</sup>
State III	70.5±5.4	99.0±6.4 <sup>d</sup>	67.8±7.0	65.1±7.7	42.0±3.8	61.9±6.2 <sup>e</sup>	54.7±4.0	52.6±3.9
RCR	4.79±0.29	10.70±1.33 <sup>d</sup>	3.71±0.18 <sup>b</sup>	2.66±0.48 <sup>b</sup>	3.69±0.27 <sup>a</sup>	10.43±1.25 <sup>d</sup>	2.71±0.20 <sup>ab</sup>	2.24±0.15 <sup>ab</sup>
FCCP	65.4±4.1	95.8±6.1 <sup>e</sup>	59.1±7.6	58.3±7.4	34.1±2.3	52.5±6.3 <sup>d</sup>	42.9±5.5	38.3±2.4

Values are means ± SE for 4–6 determinations on as many different mitochondrial preparations. Measurements were made in absence or presence of free fatty acids (FFA, using a 1 mM diluted ethanol solution) and fatty acid-free bovine serum albumin (FAF-BSA) in medium. For measurement of state IV respiration, succinate (5 mM) was added. State III respiration was initiated by addition of ADP (100 μM). RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-stimulated respiration; TN, control; GT, glucagon-treated ducklings. <sup>a</sup> *P* < 0.05, significant effect of chronic glucagon treatment; <sup>b</sup> *P* < 0.05 and <sup>c</sup> *P* < 0.01, significant effect of FFA; <sup>d</sup> *P* < 0.05 and <sup>e</sup> *P* < 0.01, significant effect of addition of FAF-BSA in respiratory medium.

TABLE 3. *Respiration of gastrocnemius muscle mitochondria and liver mitochondria as a function of FFA concentration*

FFA, nmol/ml:	Respiration, nmol O · min <sup>-1</sup> · mg protein <sup>-1</sup>							
	TN				GT			
	0	1.5	5	10	0	1.5	5	10
Gastrocnemius muscle								
Intermyofibrillar mitochondria								
Basal	21.4±2.3	20.4±2.6	21.2±2.2	22.6±3.1	28.0±7.7	28.6±7.1	28.9±7.2	25.9±6.2
+FFA	21.4±2.3	25.8±3.2*	29.3±1.3	35.3±2.6	28.0±7.7	34.5±8.4†	46.0±7.9	48.6±7.9
+FCCP	47.2±15.1	37.1±7.7	40.7±9.6	37.3±5.1	36.7±11.2	35.6±5.9	39.5±4.9	41.0±5.2
Subsarcolemmal mitochondria								
Basal	14.7±2.1	14.1±1.9	14.1±1.4	16.8±1.8	11.3±0.3	11.1±0.5	11.6±0.8	10.6±0.6
+FFA	14.7±2.1	14.1±1.4	19.0±1.1†	23.6±2.4	11.3±0.3	12.2±0.4	15.1±0.7‡	19.8±0.8
+FCCP	24.7±8.5	27.6±9.4	30.1±8.5	32.2±6.5	11.4±0.6	12.9±0.3	15.4±0.2	17.0±0.9
Liver								
Liver mitochondria								
Basal	17.4±1.8	19.3±0.9		18.0±1.5	10.6±1.8	8.9±1.1		11.4±1.8
+FFA	17.4±1.8	16.6±2.2		23.4±1.6	10.6±1.8	10.5±2.1		22.4±4.7*
+FCCP	72.5±8.9	56.0±10.7		56.8±7.1	32.4±5.6	24.9±6.6		25.4±3.6

Values are means ± SE for 4–6 determinations. To measure sensitivity to uncoupling effect of free fatty acids (FFA), respiratory rates were determined as described in MATERIALS AND METHODS. First, basal rate of respiration (basal) was determined for 3 min; second, after addition of FFA (Na palmitate diluted in ethanol), rate of respiration in presence of FFA (+FFA) was determined for 3 min; and third, after addition of 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), fully uncoupled rate (+FCCP) was determined. TN, control; GT, glucagon-treated ducklings. \* *P* < 0.05; † *P* < 0.01; and ‡ *P* < 0.001, significant effect of FFA from this concentration.

tochondria. After 6 min, the mitochondrial uncoupler FCCP was added to elicit the maximal respiratory response. The maximal FCCP-stimulated respiration decreased with increasing addition of FFA (because the maximal respiratory response was already elicited by the FFA addition).

*Oxidative and phosphorylative capacity* (Table 4). The oxidative capacity (measured as cytochrome oxidase activity in nmol O · min<sup>-1</sup> · mg mitochondrial protein<sup>-1</sup>) was higher in both intermyofibrillar and subsarcolemmal mitochondria from GT ducklings than from controls. Phos-

phorylative capacity (measured as creatine kinase activity) was lower in subsarcolemmal than in intermyofibrillar mitochondria. In the subsarcolemmal mitochondria from GT ducklings only, this activity was significantly lower than in controls. Cytochrome oxidase activity did not change in liver mitochondria. But, in consequence of the increase in mitochondrial protein mass in liver mitochondria from GT ducklings (Table 1), the organ oxidative capacity (in nmol O · min<sup>-1</sup> · g tissue<sup>-1</sup>) also increased in liver after the chronic glucagon treatment.

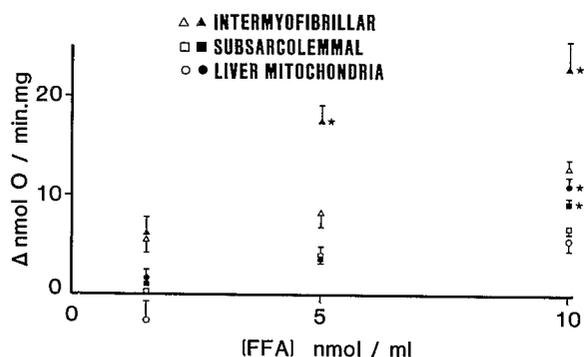


FIG. 2. Increase in respiratory rates of subsarcolemmal and intermyofibrillar muscle mitochondria and of liver mitochondria from controls and glucagon-treated ducklings. Increases were caused by addition of free fatty acids (FFA) as a function of FFA concentration and were obtained as difference between respiratory rate after addition of FFA and basal respiratory rate. Values are means  $\pm$  SE for 4–6 determinations. \*  $P < 0.05$ , significant effect of glucagon treatment.

Creatine kinase activity in liver mitochondria did not change after glucagon treatment in ducklings.

#### DISCUSSION

Chronic glucagon treatment induces in ducklings the development of thermogenic and hyperthermic responses to the injection of this hormone (6). These responses do not involve electrical muscle activity and resemble the NST observed in long-lasting cold-acclimated ducklings (5). The purposes of the present study were 1) to determine the possible mechanism of the NST measured after a glucagon test injection (6) and the adaptive enzymatic modifications responsible for the development of this thermogenic capacity and 2) to locate the tissular site of these modifications in chronic GT ducklings.

**FFA and control of thermogenesis.** FFA released by glucagon lipolysis in birds (17) may act both as a substrate for thermogenesis and as an uncoupler for mitochondria as was put forward in BAT from cold-acclimated mammals (13, 14, 19, 29, 30, 37). Under our first experimental conditions (Table 2), FFA did not act as a substrate but as an uncoupler only (decrease in RCR because of FFA). A difference in sensitivity to FFA was only found between intermyofibrillar mitochondria from GT and TN ducklings, and FFA might appear to be lacking in specificity as it had been considered previously

in BAT (23, 27). When the uncoupling effect of FFA was measured specifically under our second experimental conditions (Table 3, Fig. 2), the increased sensitivity to FFA of muscle and liver mitochondria from GT ducklings was obvious when compared with that from TN ducklings. A comparable mechanism of uncoupling was previously found in muscle mitochondria from cold-acclimated ducklings (9). But the increase in plasma (and probably in the tissues) of FFA concentrations may be responsible for a more pronounced loose coupling of mitochondria in vivo as was observed in vitro.

The powerful thermogenesis triggered by glucagon test injections may result from the uncoupling action of FFA released by this hormone strongly lipolytic in birds (17), the level of which was increased in response to cold exposure (8). The increased sensitivity of mitochondria from GT ducklings to the uncoupling effect of FFA may be attributed to the chronic glucagon treatment that may exert hormonal control similar to that of norepinephrine in mammals. However, the mechanism of muscle mitochondria uncoupling by FFA is probably different from that described for BAT mitochondria. No thermogenin or GDP binding site was observed or hypothesized in muscle and liver mitochondria, but other patterns of uncoupling have been described. Akhmerov (1) observed an ADP-independent (noncoupled with ATP synthesis) respiration in populations of heart muscle mitochondria. On the other hand, in beef heart submitochondrial particles (33) and in rat liver mitochondria (32), FFA are shown to effect a variety of uncoupling termed "decoupling." In these submitochondrial particles and mitochondria, FFA do not stimulate state III respiration as in those of muscle and liver from ducklings (Table 2). Such a decoupling effect involves a direct intermembranal proton transfer in the  $F_0$  part of the ATP synthase complex (32). A particular feature of this decoupling by FFA is the absence of significant reduction of the proton electrochemical potential gradient in contrast to classical uncouplers. It is interesting to note that muscle mitochondria from cold-acclimated ducklings show a reduction of coupling without significant modification of mitochondrial membrane potential (9).

**An adaptive enzyme modification.** The enhanced capacity for thermogenesis shown by GT ducklings should be accompanied by an increase in activity of the energy-

TABLE 4. Cytochrome oxidase and creatine kinase activity of gastrocnemius muscle mitochondria and liver mitochondria from glucagon-treated and control ducklings

	Cytochrome Oxidase Activity						Creatine Kinase Activity					
	nmol O · min <sup>-1</sup> · mg mitochondrial protein <sup>-1</sup>			nmol O · min <sup>-1</sup> · g tissue <sup>-1</sup>			U/mg protein			U/g tissue		
	TN	GT	% Diff	TN	GT	% Diff	TN	GT	% Diff	TN	GT	% Diff
Gastrocnemius muscle												
Subsarcolemmal mitochondria	633 $\pm$ 36	893 $\pm$ 79*	+41	1,004 $\pm$ 50	1,982 $\pm$ 175†	+97	1.46 $\pm$ 0.04	1.22 $\pm$ 0.03†	-16	2.34 $\pm$ 0.14	2.72 $\pm$ 0.14	
Intermyofibrillar mitochondria	703 $\pm$ 28	1,076 $\pm$ 151*	+53	4,646 $\pm$ 476	6,864 $\pm$ 617*	+48	1.94 $\pm$ 0.08‡	1.76 $\pm$ 0.07‡		12.64 $\pm$ 1.05	12.44 $\pm$ 1.00	
Liver mitochondria	279 $\pm$ 21	330 $\pm$ 29		2,102 $\pm$ 136	4,498 $\pm$ 270†	+114	0.32 $\pm$ 0.05	0.35 $\pm$ 0.02		3.75 $\pm$ 0.78	4.63 $\pm$ 0.27	

Values are means  $\pm$  SE for 6 determinations. %Diff, % difference. Comparisons are made between control (TN) and glucagon-treated (GT) ducklings (\*  $P < 0.05$ , †  $P < 0.001$ ) and between subsarcolemmal and intermyofibrillar mitochondria in each TN and GT group (‡  $P < 0.001$ ).

release enzymes in the most thermogenic tissues. Cytochrome oxidase activity was generally used as an index of the aerobic oxidative capacity of mitochondria or tissue (21). The cytochrome oxidase activity per unit weight of the gastrocnemius muscle from the GT ducklings was 57% higher than that of the controls. This increase, although independent of any sustained shivering activity, was strictly comparable to that of the pectoral (+53 and +195%) and the gastrocnemius muscles (+58 and +33%) from cold-acclimated Bantam chicks (3) and ducklings (4), respectively. On the other hand, the increase in this enzyme activity in liver was more pronounced in GT ducklings (+114%; this study) than in cold-acclimated Bantam chicks (only +19%; Ref. 3) and cold-acclimated ducklings (+47%; Ref. 4), respectively.

In muscle, mitochondrial creatine kinase activity was considered an excellent phosphorylative index (20), since the creatine phosphate shuttle theory suggested that the function of this enzyme is to synthesize creatine phosphate from creatine and ATP generated *de novo* and at the same time to return ADP to the respiratory system, thereby stimulating oxidative phosphorylation (11, 12). Because of this functional relationship between mitochondrial creatine kinase activity and oxidative phosphorylation in muscle, this enzyme activity appeared to be a good indicator of the coupling state of the muscle mitochondria, decreasing in breast muscle mitochondria from dystrophic chicken (10) or increasing in chronically stimulated fast-twitch muscle from rabbit (34) and human gastrocnemius muscle from long distance runners (2).

No change or even a decrease in phosphorylative capacity was observed in muscle mitochondria from GT ducklings in comparison with controls. A significant decrease in creatine kinase activity of subsarcolemmal mitochondria was observed as a result of chronic glucagon treatment. In liver, no difference was observed between both groups.

The discrepancy between evolution of both enzyme activities, increase in cytochrome oxidase activity and constancy or even decrease in creatine kinase activity (particularly in subsarcolemmal mitochondria), proceeds in keeping with the loose-coupled state of GT duckling mitochondria. The increased thermogenesis maintained by glucagon treatment was responsible for the respiratory enzyme development, whereas the absence of mechanical muscle activity did not require more phosphorylative activity. The lack of development of this enzyme during adaptation would indicate that an increase in the capacity for energy transfer on the creatine phosphate shuttle was not required in parallel with the adaptation of the oxidative process. It must be noted that the main process concerned in this adaptation is probably the coupling between oxidation and phosphorylation itself and not the creatine phosphate shuttle; this latter is simply deprived of its usual supply of fuel.

*Site of thermogenesis.* Because birds are devoid of BAT (5, 22), the liver and skeletal muscle in particular would be possible sites of such NST. Indeed, in cold-acclimated ducklings, skeletal muscle presents a potentiated shiv-

ering thermogenesis (PST) (7) accompanied by modifications of mitochondrial respiration, their mitochondria becoming loose coupled and more sensitive to FFA *in vitro* (9). According to the comparison between GT and TN ducklings and the increase in oxidative capacity of the mitochondria (in  $\text{nmol O} \cdot \text{min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}$ ; Table 4), only muscle appeared able to produce enhanced thermogenesis. But, on the basis of the changes in mitochondrial protein content and consequently on the changes in oxidative capacity of the tissues (in  $\text{nmol O} \cdot \text{min}^{-1} \cdot \text{g tissue}^{-1}$ ; Table 4), it is apparent that liver may also contribute to this NST. In mammals, loose-coupled muscle mitochondria have been observed in cold-acclimated white mice (35) and fur seal pups (18). In birds after cold acclimation, an increased oxidative capacity of skeletal muscles (3, 4, 9) has been shown, but no modification of phosphorylative activity has as yet been looked for, and this activity remains to be investigated in cold-acclimated ducklings.

The thermogenic and hyperthermic syndrome after chronic glucagon treatment, which artificially mimicked and amplified the effects of cold acclimation in ducklings, bears, at least in part, on a similar mechanism of mitochondrial loose coupling. In birds, liver and skeletal muscle appear to be the main sites of a glucagon-controlled NST, preserving perhaps the contractile function. Apparently, this energy-dissipating process does not hamper the contractile function, in particular if it is located on liver and subsarcolemmal mitochondria. However, it must be noted that in the first stage of true cold acclimation, shivering activity itself appears to be subject to a reduction of work efficiency under the form of PST.

In conclusion, both direct evidence based on uncoupling data and indirect evidence based on enzyme adaptation allow for ascribing at least part of the glucagon-mediated NST to liver and muscle thermogenic mitochondria. The PST and NST previously observed in cold-acclimated ducklings may also be ascribed to a similar mechanism.

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#### REFERENCES

1. AKHMEROV, R. N. Qualitative difference in mitochondria of endothermic and ectothermic animals. *FEBS Lett.* 198: 251-255, 1986.
2. APPLE, F. S., AND M. A. ROGERS. Mitochondrial creatine kinase activity alterations in skeletal muscle during long-distance running. *J. Appl. Physiol.* 61: 482-485, 1986.
3. AULIE, A., AND H. J. GRAV. Effect of cold acclimation on the oxidative capacity of skeletal muscles and liver in young bantam chicks. *Comp. Biochem. Physiol. A Comp. Physiol.* 62: 335-338, 1979.
4. BARRÉ, H., L. BAILLY, AND J. L. ROUANET. Increased oxidative capacity in skeletal muscles from cold-acclimated ducklings: a comparison with rats. *Comp. Biochem. Physiol. B Comp. Biochem.* 88: 519-522, 1987.

5. BARRÉ, H., F. COHEN-ADAD, C. DUCHAMP, AND J. L. ROUANET. Multilocular adipocytes from Muscovy ducklings differentiated in response to cold acclimation. *J. Physiol. Lond.* 375: 27-38, 1986.
6. BARRÉ, H., F. COHEN-ADAD, AND J. L. ROUANET. Two daily glucagon injections induce nonshivering thermogenesis in Muscovy ducklings. *Am. J. Physiol.* 252 (*Endocrinol. Metab.* 15): E616-E620, 1987.
7. BARRÉ, H., A. GELOEN, J. CHATONNET, A. DITTMAR, AND J. L. ROUANET. Potentiated muscular thermogenesis in cold-acclimated Muscovy duckling. *Am. J. Physiol.* 249 (*Regulatory Integrative Comp. Physiol.* 18): R533-R538, 1985.
8. BARRÉ, H., A. GELOEN, P. MIALHE, AND J. L. ROUANET. Effects of glucagon on bird thermogenesis. In: *Endocrine Regulations as Adaptive Mechanisms to the Environment*, edited by I. Assenmacher and J. Boissin. Paris: CNRS, 1986, p. 395-401.
9. BARRÉ, H., J. NEDERGAARD, AND B. CANNON. Increased respiration in skeletal muscle mitochondria from cold-acclimated ducklings: uncoupling effects of free fatty acids. *Comp. Biochem. Physiol. B Comp. Biochem.* 85: 343-348, 1986.
10. BENNETT, V. D., N. HALL, M. DELUCA, AND C. H. SUELTER. Decreased mitochondrial creatine kinase activity in dystrophic chicken breast muscle alters creatine-linked respiratory coupling. *Arch. Biochem. Biophys.* 240: 380-391, 1985.
11. BESSMAN, S. P., AND C. L. CARPENTER. The creatine-creatine phosphate energy shuttle. *Annu. Rev. Biochem.* 54: 831-862, 1985.
12. BESSMAN, S. P., AND A. FONYO. The possible role of the mitochondrial bound creatine kinase in regulation of mitochondrial respiration. *Biochem. Biophys. Res. Commun.* 22: 597-602, 1966.
13. BULYCHEV, A., R. KRAMAR, Z. DRAHOTA, AND O. LINDBERG. Role of specific endogenous fatty acid fraction in the coupling-uncoupling mechanism of oxidative phosphorylation of brown adipose tissue. *Exp. Cell Res.* 72: 169-187, 1972.
14. CANNON, B., D. G. NICHOLLS, AND O. LINDBERG. Purine nucleotides and fatty acids in energy coupling of mitochondria from brown adipose tissue. In: *Mechanisms in Bioenergetics*, edited by G. F. Azzone, L. Ernster, S. Papa, E. Quagliariello, and N. Sili-prand. New York: Academic, 1973, p. 357-364.
15. ESTABROOK, R. W. Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. In: *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan. New York: Academic, 1967, vol. X, p. 41-47.
16. FOSTER, G., E. BERNT, AND U. BERGMAYER. Creatine kinase. In: *Methods of Enzymatic Analysis*, edited by H. U. Bergmeyer. New York: Academic, 1974, p. 784-793.
17. GRANDE, F., AND W. F. PRIGGE. Glucagon infusion, plasma FFA, and triglycerides, blood sugar, and liver lipids in birds. *Am. J. Physiol.* 218: 1406-1411, 1970.
18. GRAV, H. J., AND A. S. BLIX. A source of non-shivering thermogenesis in fur seal skeletal muscle. *Science Wash. DC* 204: 87-89, 1979.
19. HITTELMAN, K. J., O. LINDBERG, AND B. CANNON. Oxidative phosphorylation and compartmentation of fatty acid metabolism in brown fat mitochondria. *Eur. J. Biochem.* 11: 183-192, 1969.
20. JACOBUS, W. E., AND A. L. LEHNINGER. Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. *J. Biol. Chem.* 248: 4803-4810, 1973.
21. JANSKY, L. Nonshivering thermogenesis and its thermoregulatory significance. *Biol. Rev.* 48: 85-132, 1973.
22. JOHNSTON, D. W. The absence of brown adipose tissue in birds. *Comp. Biochem. Physiol. A Comp. Physiol.* 40: 1107-1108, 1971.
23. LINDBERG, O., B. CANNON, AND J. NEDERGAARD. Thermogenic mitochondria. In: *Mitochondria and Microsomes*, edited by C. P. Lee, G. Schatz, and G. Dallner. Reading, MA: Addison-Wesley, 1981, p. 93-119.
24. LOCKE, R. M., AND D. G. NICHOLLS. A reevaluation of the role of fatty acids in the physiological regulation of the proton conductance of brown adipose tissue mitochondria. *FEBS Lett.* 135: 249-252, 1981.
25. LOCKE, R. M., E. RIAL, I. D. SCOTT, AND D. G. NICHOLLS. Fatty acids as acute regulators of the proton conductance of hamster brown-fat mitochondria. *Eur. J. Biochem.* 129: 373-380, 1982.
26. NEDERGAARD, J., AND B. CANNON. Preparation and properties of mitochondria from different sources. In: *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan. New York: Academic, 1979, vol. LV, p. 3-28.
27. NICHOLLS, D. G. Brown adipose tissue mitochondria. *Biochim. Biophys. Acta* 549: 1-29, 1979.
28. PALMER, J. W., B. TANDLER, AND C. L. HOPPEL. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J. Biol. Chem.* 252: 8731-8739, 1977.
29. PRUSINER, S. B., B. CANNON, AND O. LINDBERG. Oxidative metabolism in cells isolated from brown adipose tissue. I. Catecholamine and fatty acid stimulation of respiration. *Eur. J. Biochem.* 6: 15-22, 1968.
30. RAFAEL, J., H. J. LUDOLPH, AND H. J. HOHORST. Mitochondrien aus braunem Fettgewebe: entkopplung der Atmungskettenphosphorylierung durch langkettige Fettsäuren und Rekopplung durch Guanositriphosphat. *Hoppe-Seyler's Z. Physiol. Chem.* 350: 1121-1131, 1969.
31. RIAL, E., A. POUSTIE, AND D. G. NICHOLLS. Brown adipose tissue mitochondria: the regulation of the 32,000-M<sub>r</sub> uncoupling protein by fatty acids and purine nucleotides. *Eur. J. Biochem.* 137: 197-203, 1983.
32. ROTTENBERG, H., AND K. HASHIMOTO. Fatty acid uncoupling of oxidative phosphorylation in rat liver mitochondria. *Biochemistry* 25: 1747-1755, 1986.
33. ROTTENBERG, H., AND S. STEINER-MORDOCH. Free fatty acids decouple oxidative phosphorylation by dissipating intramembranal protons without inhibiting ATP synthesis driven by the proton electrochemical gradient. *FEBS Lett.* 202: 314-318, 1986.
34. SCHMITT, T., AND D. PETTE. Increased mitochondrial creatine kinase in chronically stimulated fast-twitch rabbit muscle. *FEBS Lett.* 188: 341-344, 1985.
35. SKULACHEV, V. P., S. P. MASLOV, V. G. SIVKOVA, L. P. KALINICHENKO, AND G. M. MASLOVA. Uncoupling of oxidation from phosphorylation in muscles of cold-adapted white mice. *Biochemistry (Engl. Transl. Biokhimiya)* 28: 54-60, 1963.
36. WARDLAW, G. M., AND M. L. KAPLAN. Oxygen consumption and oxidative capacity of muscles from young obese and nonobese Zucker rats. *Am. J. Physiol.* 247 (*Regulatory Integrative Comp. Physiol.* 16): R911-R917, 1984.
37. WILLIAMSON, J. R. Control of energy metabolism in hamster brown adipose tissue. *J. Biol. Chem.* 245: 2043-2050, 1970.