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 thermogenic responses without shivering to glucagon test injection and in control ducklings (TN; both aged 44 ± 1 days and reared at thermoneutrality), mitochondrial respiration was shown despite a strong increase in cytochrome oxidase activity (+114% if expressed per mg mitochondrial protein 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.

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-acclimated 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-acclimatized 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 (Éts Grimaud) were used. These were fed a commercial mash (Sanders 5061) ad libitum and had free access to water. The glucagon treatment schedule described.

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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 resuspended in 20 ml of isolation buffer A and centrifuged at 8,700 g for 10 min. The resulting pellet was resuspended in 10 ml buffer B and then was 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 centrifuged 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 5.2 ± 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 MgCl₂, 5 ethylene glycol-bis(β-aminoethylether)-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 (28). 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 O₂ 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·PO₄, 20 Tris·HCl (adjusted pH 7.2), 2 MgCl₂, 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 (FAB-BSA).

**Cytochrome oxidase activity.** Cytochrome oxidase activity in muscle and liver mitochondria was determined polarographically at 25°C, using a Clark O₂ 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 NAD malonate, 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 MgCl₂, 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 O₂ consumption observed after the addition of substrate (4 mM Na-ascorbate with 0.3 mM TMPD) and mitochondria and the rate of O₂ consumption observed after the addition of substrate alone to take the autoxi-
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 β-NADH, 6.1 ATP, 1.5 phosphoenolpyruvate (PEP), 6.1 MgCl₂, 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 μg). 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 micrography. 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 Frobalo (Paris, France); lubrol was from Serva-Heidelberg (FRG); and carbonyl cyanide p-trifluoromethoxyphenyllhydrazone (FCCP) was from Fluka (Buch, Switzerland). Other chemicals were pro analysis grade.

Units and statistics. International units have been used throughout this paper. Values have been presented as means ± 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<sub>CR</sub> = 54.4, P < 0.001; F<sub>TN</sub> = 19.1, P < 0.05), 5 nmol/ml FFA in subsarcolemmal mitochondria (F<sub>GT</sub> = 31.1, P < 0.001; F<sub>FN</sub> = 13.8, P < 0.01) from both GT and TN ducklings, and 10 nmol/ml FFA in liver mitochondria (F<sub>GT</sub> = 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**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glucagon Treated</th>
<th>Variation, %</th>
</tr>
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<tbody>
<tr>
<td><strong>Body mass, g</strong></td>
<td>2,474.1±64.2</td>
<td>1,969.7±84.4</td>
<td>−20.4</td>
</tr>
<tr>
<td><strong>Liver mass, g</strong></td>
<td>66.0±2.5</td>
<td>61.2±4.8</td>
<td>+24.9</td>
</tr>
<tr>
<td><strong>Liver mass/body mass, %</strong></td>
<td>2.64±0.13</td>
<td>4.11±0.14</td>
<td></td>
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<tr>
<td><strong>Mitochondrial protein mass, mg/gww</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subsarcolemmal mitochondria</td>
<td>1.61±0.10</td>
<td>2.22±0.17†</td>
<td>+37.9</td>
</tr>
<tr>
<td>Intermyofibrillar mitochondria</td>
<td>6.61±0.66</td>
<td>7.07±0.64</td>
<td></td>
</tr>
<tr>
<td>Liver mitochondria</td>
<td>7.75±0.72</td>
<td>13.23±1.00†</td>
<td>+70.1</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 ducklings in each group. * P < 0.05, † P < 0.01, and ‡ P < 0.001.
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 O₂·min⁻¹·mg mitochondrial protein⁻¹) was higher in both intermyofibrillar and subsarcolemmal mitochondria from GT ducklings than from controls. Phosphorylative 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 O₂·min⁻¹·g tissue⁻¹) also increased in liver after the chronic glucagon treatment.

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

<table>
<thead>
<tr>
<th></th>
<th>Respiration, nmol O₂·min⁻¹·mg mitochondrial protein⁻¹</th>
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<tbody>
<tr>
<td></td>
<td>TN</td>
</tr>
<tr>
<td></td>
<td>FFA, nmol/ml:</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Gastrocnemius muscle</strong></td>
<td></td>
</tr>
<tr>
<td>Intermyofibrillar mitochondria</td>
<td></td>
</tr>
<tr>
<td>State IV</td>
<td>42.9±4.3</td>
</tr>
<tr>
<td>State III</td>
<td>200±18.1</td>
</tr>
<tr>
<td>RCR</td>
<td>4.12±0.25</td>
</tr>
<tr>
<td>FCCP</td>
<td>202±19.6</td>
</tr>
<tr>
<td><strong>Subsarcolemmal mitochondria</strong></td>
<td></td>
</tr>
<tr>
<td>State IV</td>
<td>27.7±2.8</td>
</tr>
<tr>
<td>State III</td>
<td>59.2±5.1</td>
</tr>
<tr>
<td>RCR</td>
<td>2.14±0.18</td>
</tr>
<tr>
<td>FCCP</td>
<td>59.2±5.1</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
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<tr>
<td>Liver mitochondria</td>
<td></td>
</tr>
<tr>
<td>State IV</td>
<td>14.9±1.5</td>
</tr>
<tr>
<td>State III</td>
<td>70.8±5.4</td>
</tr>
<tr>
<td>RCR</td>
<td>4.70±0.29</td>
</tr>
<tr>
<td>FCCP</td>
<td>65.4±4.1</td>
</tr>
</tbody>
</table>

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. *P < 0.05, significant effect of chronic glucagon treatment; †P < 0.05 and ‡P < 0.01, significant effect of FFA; §P < 0.01, significant effect of addition of FFA in respiratory medium.
Creative 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 muscles (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 to the injection of this hormone (6)). These responses do not involve electrical muscle activity and resemble the NST 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 intermembrane proton transfer in the F, 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-
release enzymes in the most thermogenic tissues. Cyto-
chrome 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 duck-
lings was 57% higher than that of the controls. This
increase, although independent of any sustained shiv-
ering activity, was strictly comparable to that of the pec-
toral (+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), respec-
tively.

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 phos-
phate 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 mito-
chondrial creatine kinase activity and oxidative phos-
phorylation in muscle, this enzyme activity appeared to
be a good indicator of the coupling state of the muscle
mitochondria, decreasing in breast muscle mitochondria
drom dystrophic chicken (10) or increasing in chronically
stimulated fast-twitch muscle from rabbit (34) and hu-
man gastrocnemius muscle from long distance runners
(2).

No change or even a decrease in phosphorylative ca-
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 gluca-
gon treatment. In liver, no difference was observed be-
tween 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 ducking
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 capac-
ity 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 de-
prived 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 modifi-
cations 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 nmol O.min\(^{-1}\)·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 nmol
O.min\(^{-1}\)·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 ca-
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 investi-
gated 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 mito-
chondrial loose coupling. In birds, liver and skeletal
muscle appear to be the main sites of a glucagon-con-
trolled 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. How-
ever, 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 uncou-
pling data and indirect evidence based on enzyme adap-
tation allow for ascribing at least part of the glucagon-
mediated NST to liver and muscle thermogenic mito-
chondria. 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 en-
dothermic 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 run-

3. AULIE, A., AND H. J. GRAV. Effect of cold acclimation on the
oxidative capacity of skeletal muscles and liver in young bantam
1979.

4. BARRÉ, H., L. BAILLY, AND J. L. ROUANET. Increased oxidative
capacity in skeletal muscles from cold-acclimated ducklings: a


