# Effect of hypoenergetic feeding on muscle oxidative phosphorylation and mitochondrial complex I–IV activities in rats[1–3]

*Annie G Madapallimattam, Lorraine Law, and Khursheed N Jeejeebhoy*

**ABSTRACT**

**Background:** Previous studies showed that malnutrition reduces the activity of complexes I, II, and III in the mitochondria of skeletal muscle.

**Objective:** We hypothesized that malnutrition would influence oxidative phosphorylation and mitochondrial complex activity in the skeletal muscle of rats.

**Design:** Thirty-two rats were assigned either to a control group with an ad libitum intake of 364 kJ/d or to a hypoenergetic group with an intake of 92 kJ/d. Eleven of these rats received the hypoenergetic diet for 10 d, 2 for 5 d, 2 for 6 d, 2 for 7 d, and 1 each for 8 and 11 d to achieve a distributed weight loss. Ten controls were fed for 10 d, 2 for 7 d, and 1 for 5 d, to match day 10, day 5, and the midpoint (day 7) of 6–8 d of hypoenergetic feeding. The 2 diets provided the same volume, electrolytes, vitamins, and trace elements but different amounts of energy.

**Results:** A significant relation was observed between weight loss and the state 4 and 3 oxidation rates with pyruvate + malate and for state 3 glutamate + malate and succinate + rotenone but not with tetramethyl-*p*-phenylenediamine + ascorbate + antimycin A (TMPD). Similarly, a significant relation was observed between the degree of weight loss and complex I and III activities but not with complex II and IV activities.

**Conclusions:** The complex activities of the mitochondrial oxidative phosphorylation chain in muscle were depressed selectively with energy deprivation when compared with normally fed rats. These findings may partly explain the mechanism of reduced muscle energetics in energy malnutrition. *Am J Clin Nutr* 2002;76:1031–9.

**KEY WORDS** Malnutrition, energy metabolism, oxidative phosphorylation, respiratory chain, hypoenergetic feeding, complex activity, rats

## INTRODUCTION

Hypoenergetic feeding is associated with significant changes in the force-frequency curve, the relaxation rate, and the fatigability of skeletal muscle (1). It is also associated with reduced activities of phosphofructokinase (EC 2.7.1.11), the rate-limiting enzyme of the glycolytic pathway, and of succinate dehydrogenase (EC 1.3.99.1), a rate-limiting enzyme of the Krebs cycle (1). In addition, intracellular potassium in muscle is reduced in hypoenergetically fed rats (2). Together, these findings suggest that muscle cell energetics are altered by protein-energy restriction. Consistent with this hypothesis are the findings of $^{31}$P nuclear magnetic resonance studies, which show that hypoenergetically fed growing rats (25% of the protein-energy intake of control animals resulting in a 25% weight loss over a 7-d period) have a lower ratio of phosphocreatine to ATP, a lower amount of free energy from ATP hydrolysis (delta $G_{ATP}$), and a higher concentration of free ADP in the gastrocnemius muscle than do controls (3, 4). These studies showed that rephosphorylation of ADP was slower in the muscles of hypoenergetically fed rats than in control rats (3). The abovementioned abnormalities, induced by hypoenergetic feeding, were corrected by increasing energy intakes (refeeding for 7 d). Moreover, Ardawi et al (5) showed reduced oxygen uptake by the mitochondria of hypoenergetically fed rats. Taken together, these findings support the hypothesis that mitochondrial oxidative phosphorylation is reduced in the muscle of hypoenergetically fed rats. In a previous study we showed that hypoenergetic feeding resulted in a reduction in the activity of complex I [NADH dehydrogenase (ubiquinone), EC 1.6.99.5], complex II [succinate dehydrogenase (ubiquinone), EC 1.3.5.1], and complex III (ubiquinol–cytochrome-*c* reductase, EC 1.10.2.2) in the muscle and mononuclear cells of rats (6) and in the mononuclear cells of humans. Refeeding restored the depressed mitochondrial complex activities in both rats and humans (6–8).

Mitochondria occupy a pivotal position in aerobic ATP production through the oxidative phosphorylation of ADP. All of the energy-producing reactions, including glycolysis and the tricarboxylic acid cycle, generate reducing equivalents in the form of NADH and reduced flavins ($FADH_2$), which are ultimately oxidized by oxygen through a chain of oxidoreduction reactions occurring in complexes I, II, III, and IV (cytochrome-*c* oxidase, EC 1.9.3.1)) in the inner mitochondrial membrane (oxidative phosphorylation system). Complexes I and II oxidize NADH and succinate, respectively, and the electron acceptor is coenzyme $Q_{10}$, which becomes reduced. The reduced coenzyme $Q_{10}$ is subsequently oxidized by complex III and the electron acceptor is

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**TABLE 1**
Composition of the liquid diet (per 60 mL) consumed by control and hypoenergetically fed rats[1]

| | Control rats | Hypoenergetic rats |
|---|---|---|
| | *kJ* | |
| Energy | 364.0 | 92.0 |
| D-Glucose | 262.8 | 66.0 |
| Amino acids | 48.8 | 12.5 |
| Fat | 52.4 | 13.5 |

[1] An amino acid solution similar to Travasol 10% (Baxter Corporation, Toronto) and Intralipid 20% (Clintec Nutrition Company, Mississauga, Canada) was used to make up the liquid diet. The micronutrient and vitamin content was identical in each of the 4 formulas: 74.0 mg Na, 80.7 mg K, 247.6 mg Cl, 27.7 mg Ca, 9.2 mg Mg, 0.27 mg Zn, 0.56 mg Mn, 0.11 mg Cu, 0.81 mg Fe, 2.3 μg Se, 7.0 μg Cr, 180.0 mg acetate, 18.3 mg phosphate, 6.1 μg I, 165 μg vitamin A (retinol palmitate), 30 mg vitamin C, 2.1 μg vitamin $D_3$, 0.55 mg *RRR*-α-tocopherol, 1.5 mg thiamine, 0.3 mg riboflavin, 3.0 mg niacinamide, 0.36 mg pyridoxine, and 0.78 pantothenate.

cytochrome *c*, which is reduced. Complex IV facilitates the oxidation of reduced cytochrome *c* by using oxygen to form oxidized cytochrome *c* and water. These processes create a proton gradient across the inner mitochondrial membrane, which is used to drive ATP synthesis by complex V ($F0F_1$ ATPase) (9). Although reduced complex activity probably reduces oxidative phosphorylation, no studies have examined the effect of hypoenergetic feeding on the different paths of oxidative phosphorylation and compared these changes with complex activity.

The purpose of the present investigation was to establish whether nutritional manipulation reduces oxidative phosphorylation through complexes I, II, III, and IV and to correlate these results with changes in mitochondrial complex (I–IV) activities in the gastrocnemius muscle.

## MATERIALS AND METHODS

### Animals

Male Wistar rats initially weighing between 250 and 260 g (8 wk of age) were obtained from Charles River Canada Inc (Montreal) and housed individually in wire cages in a 12-h light-dark controlled room at a temperature of 22 °C. On arrival at the animal facility, the rats were acclimatized for ≥2 d, during which time they were fed a control liquid diet (**Table 1**) before the start of the study. The rats were then randomly assigned to either the control fed (CF) group or the hypoenergetically fed (HF) group. The diets used were identical to those published previously (6). The rats in the CF group ($n$ = 13) consumed the control diet (Table 1) ad libitum for a duration matched with that for the rats fed hypoenergetically. On average, the ad libitum consumption was 60 mL/d, which provided an energy intake of 364 kJ/d. The nutrient content of the control liquid formula met the recommended requirements for rats and was previously shown to result in a weight gain comparable with that in rats fed Purina rodent chow 5001(Ralston Purina Corp, Strathnoy, Canada). In addition, we had previously confirmed that this formula sustains weight gain and normal body composition when provided for 2 mo (data not shown). The rats in the HF group ($n$ = 19) consumed the same volume of 60 mL/d, but the hypoenergetic diet provided 92 kJ/d. Eleven rats were fed this diet for 10 d (Hypo10). In addition, to obtain a wide distribution of weight losses, the rats were also fed this diet for 5 ($n$ = 2), 6 ($n$ = 2), 7 ($n$ = 2), 8 ($n$ = 1), or 11 ($n$ = 1) d. Ten CF rats were fed for 10 d, 2 for 7 d, and 1 for 5 d, to match day 10, day 5, and the midpoint of 6–8 d of hypoenergetic feeding. The 2 diets contained the same amount of electrolytes, vitamins, and trace elements but different amounts of energy. The protocol was approved by the University of Toronto Animal Care Committee.

### Preparation of muscle homogenate

Under general anesthesia, the gastrocnemius muscle was excised and the animals were killed. The muscle was trimmed of fat and connective tissue and immediately placed in ice-cold buffer A (100 mmol KCl/L, 5 mmol $MgSO_4 \cdot 7H_2O$/L, 5 mmol EDTA/L, and 50 mmol tris/L; pH 7.4). The muscle was homogenized in buffer A with added 1 mmol ATP/L with the use of a Polytron (model PT 1200; Kinematica AG, Littau-Lucerne, Switzerland) homogenizer for 10 s on full power in batches of 0.5 g muscle/tube. We found that the Polytron homogenization gave a better yield of undamaged mitochondria than did a polytetrafluoroethylene homogenizer. Our observations with the Polytron were similar to those published previously by Palmer et al (10). The homogenate was made up to 20 volumes with respect to the original wet weight of tissue with buffer A with added 1 mmol ATP/L.

### Preparation of skeletal muscle mitochondria

The homogenate was centrifuged at 600 × *g* for 10 min at 4 °C and the supernatant fluid put aside. The pellet was resuspended in 5 volumes of the above buffer and recentrifuged at 600 × *g* for 10 min at 4 °C. The supernatant fluids were combined, diluted 5-fold with the above buffer, and filtered through 4 layers of cheesecloth to remove coarse debris. The filtered supernatant fluids were then centrifuged at 3000 × *g* for 10 min at 4 °C, and the crude mitochondrial pellet was resuspended in 3 mL of the above buffer. An aliquot was removed for protein measurement. The resuspended pellet was recentrifuged at 3000 × *g* for 10 min at 4 °C. The purified pellet was suspended in a small volume of resuspension buffer [100 mmol KCl/L, 10 mmol MOPS/L, and 0.2% fat-free bovine serum albumin (BSA); pH 7.4] for a final concentration of 10 mg protein/mL. Aliquots of this pellet were used for polarographic measurement of respiration and enzyme complex activity by using the methods described below.

### Protein assay

The protein content of the mitochondria was determined with the method of Lowry et al (11) by using appropriate blanks.

### Polarographic measurement of oxygen consumption

The respiratory activities of mitochondria from muscle biopsy samples were assayed soon after isolation, polarographically, with the use of a modified version of the method described by Estabrook (12). Mitochondrial respiratory rates were measured at 30 °C in a thermostatically controlled micro oxygen chamber (YSI 5356; Yellow Springs Inc, Yellow Springs, OH) equipped with a micro oxygen probe (YSI 5357) coated with a standard membrane (YSI 5775); 1.9 mL buffer (250 mmol sucrose/L, 50 mmol KCl/L, 25 mmol tris/L, and 10 mmol $K_2HPO_4$/L; pH 7.4) was equilibrated with the oxygen electrode for 3 min with stirring. Freshly prepared mitochondria (0.8–1.0 mg protein) were then added to the buffer in the chamber and incubated for another 3 min with

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stirring. Respiration was started by adding one of the respiratory substrates at a final concentration of pyruvate (20 mmol/L) + malate (10 mmol/L), glutamate (5 mmol/L) + malate (2.5 mmol/L), succinate (20 mmol/L) + rotenone (2 g/mL), and TMPD (0.25 mmol/L) + ascorbate (2 mmol/L) + antimycin A (2 g/mL) to separate aliquots of each mitochondrial preparation. The addition of a respiratory substrate allowed the measurement of coupled state 4 respiration, and was followed by the addition of 500 nmol ADP to obtain the coupled state 3 respiratory rate and the respective moles of ATP phosphorous generated per mole of oxygen consumed (P:O) and the respiratory control ratio (RCR). These additions allowed the following paths of oxidative phosphorylation to be studied.

*Pyruvate + malate*

The glycolytic product pyruvate is converted by oxidative decarboxylation to acetyl CoA by pyruvate dehydrogenase (EC 1.2.2.2). Acetyl CoA subsequently enters the citric acid cycle, and the reducing equivalents generated enter the electron transport chain and are oxidized by passage through complexes I, III, and IV.

*Glutamate + malate*

Arginine, glutamine, histidine, and proline are all degraded by conversion to glutamate, which in turn is oxidized to α-ketoglutarate by glutamate dehydrogenase (EC 1.4.1.2). α-Ketoglutarate enters the citric acid cycle and the reducing equivalents generated enter the electron transport chain and are oxidized by passage through complexes I, III, and IV.

*Succinate + rotenone*

The addition of rotenone stops oxidation by complex I. Succinate enters the electron transport chain after the rotenone-blocked step and is oxidized by passage through complexes II, III, and IV.

*TMPD + ascorbate + antimycin A*

Addition of antimycin A inhibits complex III. TMPD is an ascorbate-reducible redox carrier that transfers electrons directly to cytochrome *c*. Addition of TMPD and ascorbate to the antimycin A–inhibited reaction mixture results in resumption of oxygen consumption through complex IV.

**Spectrophotometric measurement of complex enzyme activity**

*Citrate synthase*

Citrate synthase (EC 4.1.3.7) was measured by the method of Robinson et al (13). Mitochondria were diluted 5-fold in 20 mmol $KPO_4$ buffer/L (pH 7.2) to a final concentration of 2 mg/mL. The mitochondrial membrane was disrupted by freezing and thawing 3 times in 20 mmol $KPO_4$ buffer (pH 7.2) to facilitate access of NADH to its binding sites on the inner aspect of the inner mitochondrial membrane. Mitochondria were then further solubilized by adding an equal amount of 0.2% Triton (Sigma-Aldrich Corp, St Louis).

This method is based on the chemical coupling of the reduced form of coenzyme A (CoASH), released from acetyl-CoA during the enzymatic synthesis of citrate, to Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid). The release of the absorbing mercaptide ion is followed at 412 nm, with 530 nm as the reference. The solubilized mitochondria (10 μg) were added to a reaction mixture containing 0.1 mol tris-HCl buffer/L (pH 8.0), 0.25 mmol oxaloacetate/L, 0.1 mmol 5,5′-dithiobis-(2-nitrobenzoic acid)/L, and 0.05 mmol acetyl CoA/L. The reaction was carried out at 25 °C and followed for 2–3 min. An extinction coefficient of 13.6 $mmol \cdot L^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

*Activity of complex I*

Complex I activity was measured by following the decrease in absorbance due to the oxidation of NADH at 340 nm with the reference set at 425 nm (14). The mitochondria (40 μg) were disrupted by freezing and thawing 3 times in 20 mmol potassium phosphate buffer/L (pH 7.2) and then they were added to a buffer containing 25 mmol potassium phosphate/L (pH 7.2), 5 mmol $MgCl_2$/L, 2 mmol KCN/L (to inhibit complex IV), 2 mg antimycin A/mL (to inhibit complex III), 2.5 mg fat-free BSA/mL, 0.13 mmol NADH/L, and 65 μmol ubiquinone/L. The NADH-ubiquinone oxidoreductase activity was measured for 3–5 min and then again for another 3 min after 2 mg rotenone was added. The decrease in NADH activity after rotenone was added is the blank slope and was subtracted from the NADH slope before adding rotenone. An extinction coefficient of 6.22 $mmol \cdot L^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

*Activity of complex II*

Complex II activity was measured by following the reduction of 2,6-dichlorophenolindophenol at 600 nm (14). The mitochondria (40 μg) were disrupted as above, preincubated in a buffer containing 25 mmol potassium phosphate/L (pH 7.2), 5 mmol $MgCl_2$/L, and 20 mmol succinate/L at 30 °C for 10 min to fully activate the enzyme. After preincubation, 10 mmol KCN/L (to inhibit complex IV), 2 mg antimycin A/mL (to inhibit complex III), 2 mg rotenone/mL (to inhibit complex I), and 50 μmol 2,6-dichlorophenolindophenol/L were added, and baseline changes were recorded; 16 mg ubiquinone was then added and the reduction was measured. An extinction coefficient of 19.1 $mmol \cdot L^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

*Activity of complex III*

Complex III activity was measured by following the reduction of cytochrome *c* $Fe^{3+}$ by decyl ubiquinol (15). The mitochondria (10 μg) were disrupted and solubilized in 0.1% Triton and were added to an incubation mixture containing 100 μmol EDTA/L, 0.2% fat-free BSA, 3 mmol sodium azide/L, and 60 μmol cytochrome *c* $Fe^{3+}$/L in 50 mmol potassium phosphate buffer/L (pH 8.0) in a reaction volume of 1 mL. The mixture was preincubated for 3 min at room temperature. The reaction was started by adding 10 μL decyl ubiquinol and the decrease in absorbance at 550 nm was followed. The blank was measured by adding 10 mg antimycin A to the above mixture to inhibit complex III. An extinction coefficient of 21 $mmol \cdot L^{-1} \cdot cm^{-1}$ was used to calculate absolute changes.

*Activity of complex IV*

Complex IV activity was measured by following the oxidation of cytochrome *c* $Fe^{2+}$ (16, 17). The mitochondria were disrupted by freezing and thawing them 3 times in 20 mmol potassium phosphate buffer/L (pH 7.2) and were extracted in 1.5% (wt:vol) dodecyl maltoside. Extracted mitochondrial protein (10 μg) was added to 1 mL of a reaction mixture consisting of 50 mmol phosphate buffer/L (pH 7.2) and 0.05% dodecyl maltoside. The reaction was started by adding cytochrome *c* $Fe^{2+}$ to a final concentration of

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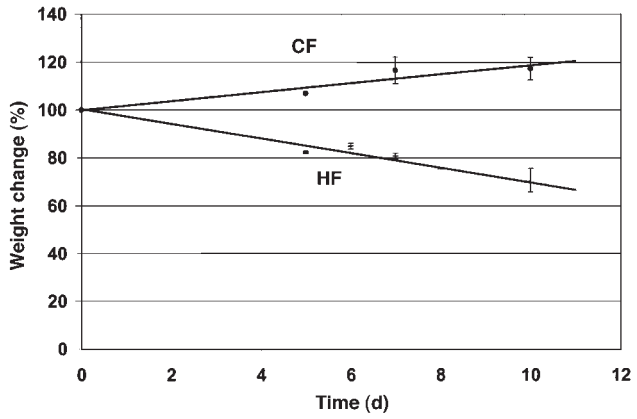


**FIGURE 1.** Mean (±SEM) percentage weight change in control-fed (CF) and hypoenergetically fed (HF) rats from baseline (day 0) at the start of the diets. CF: 10 d ($n$ = 10), 7 d ($n$ = 2), and 5 d ($n$ = 1). HF: 10 d ($n$ = 11), 7 d ($n$ = 2), 6 d ($n$ = 2), 5 d ($n$ = 2), and 8 and 11 d ($n$ = 1). The data points with no error bars represent single animals.

15 μmol/L. The reaction was followed at an absorbance of 550 nm with the reference at 580 nm for 2 min. After 2 min, the remaining unoxidized cytochrome $c$ $Fe^{2+}$ in the reaction mixture was completely oxidized by adding saturated potassium ferricyanide. The optical density was again measured after complete oxidation. The first-order rate constant for oxidation was calculated from the initial rate and total cytochrome $c$.

### Chemicals

Ubiquinone [500 mg/500 μL (4 mol/L)] was diluted in ethanol before use. All other chemicals were purchased from Sigma Chemical Co (St Louis).

### Statistical analysis

The results for the CF group fed for 10 d (CF10) and for the HF10 group were expressed as means ± SEMs. The differences between the CF10 and HF10 groups were tested by the Kruskal-Wallis nonparametric test. The relation of weight loss to oxidative phosphorylation and complex activity was studied by testing linear and logarithmic regressions for significance by using STATVIEW 5 (SAS Institute Inc, Cary, NC).

## RESULTS

### Weight change

The CF group gained weight, and the HF group lost weight. The weight loss was progressive with the duration of hypoenergetic feeding (**Figure 1**). The mean weight gain for the CF10 group was 17.5 ± 4%, and the mean weight loss for the HF10 group was 29.2 ± 4.1% of the weight at the start of the dietary treatment ($P < 0.001$).

### Effect of hypoenergetic feeding on respiratory rates

The state 4 respiratory rate after 10 d of hypoenergetic feeding was significantly lower than ($P < 0.05$) after the control feeding, with pyruvate + malate being 8.9 ± 0.8 and 10.85 ± 1.1 nmol · $min^{-1}$ · mg mitochondrial $protein^{-1}$, respectively. There was a weak but significant linear correlation between state 4 respiration with pyruvate + malate and percentage weight loss ($F = 5.31$; df = 1, 29; $P < 0.03$). The state 4 respiratory rates in the HF10 group for glutamate + malate (10.5 ± 0.9 and 11.7 ± 1.1 nmol · $min^{-1}$ · mg mitochondrial $protein^{-1}$, respectively) and succinate + rotenone (13.1 ± 1.1 and 16 ± 1.3 nmol · $min^{-1}$ · mg mitochondrial $protein^{-1}$, respectively) were also lower than those in the CF10 group but not significantly so, and the regressions between the state 4 respiratory rates and percentage weight loss for these substrates were not significant.

The state 3 coupled respiratory rates measured with the 4 substrates are plotted as a function of the percentage weight change with hypoenergetic feeding in **Figure 2**. To test the fit of the nonlinear functions, including that of a logarithmic one, weight changes were normalized to a positive number by adding 40. The addition of 40 is a fixed offset to make all data positive to avoid errors from taking the logarithms of a negative number. Because it is fixed offset, the animals with the greatest weight loss had the lowest percentage weight change. The statistical analysis of the data, carried out by plotting the measured respiratory rates as a function of the percentage of normalized weight change, showed a significant correlation between the respiratory rates measured with glutamate + malate, pyruvate + malate, and succinate + rotenone and the percentage of normalized weight change (Figure 2). Both the linear (data not given) and logarithmic analyses gave a significant fit, but the logarithmic plot was the better fit. However, the type of fit does not imply a specific biological relation. Oxygen consumption after the addition of TMPD + ascorbate + antimycin A was not significantly related to weight change.

### Effect of hypoenergetic feeding on the RCR, state 3 and oxidative phosphorylation rates, and the P:O

The state 3 and oxidative phosphorylation rates after 10 d of feeding were significantly lower in the HF group than in the CF group (**Figure 3**). However, hypoenergetic feeding did not alter the P:O. In addition, the RCR was significantly lower after 10 d of hypoenergetic feeding than after a control diet.

### Effect of hypoenergetic feeding on complex activity

The activity of mitochondrial citrate synthase and of complexes I, II, III, and IV correlated with the percentage weight change (**Figure 4**). The statistical analysis of the data was carried out by plotting the measured enzyme complex activities as a function of the percentage of normalized weight change. Although both the linear (data not given) and logarithmic analyses gave a significant fit, the logarithmic plot was the better fit. However, the type of fit does not imply a specific biological relation. The correlation between percentage weight change and activities of complexes I and III were highly significant. In contrast, the correlation with complex IV was poor and was significant (barely) only for the logarithmic plot (data not given). Finally, the correlation with complex II activity was not significant in either plot. However, the lack of relation may have been due to the limited sample size. The activities of complex I and complex III after 10 d of hypoenergetic feeding were significantly lower than those after the control feeding (**Figure 5**).

### Relation between state 3 respiratory activity and complex activity

There was a significant relation between state 3 respiratory activity and the activities of complex I and III (**Figure 6**).

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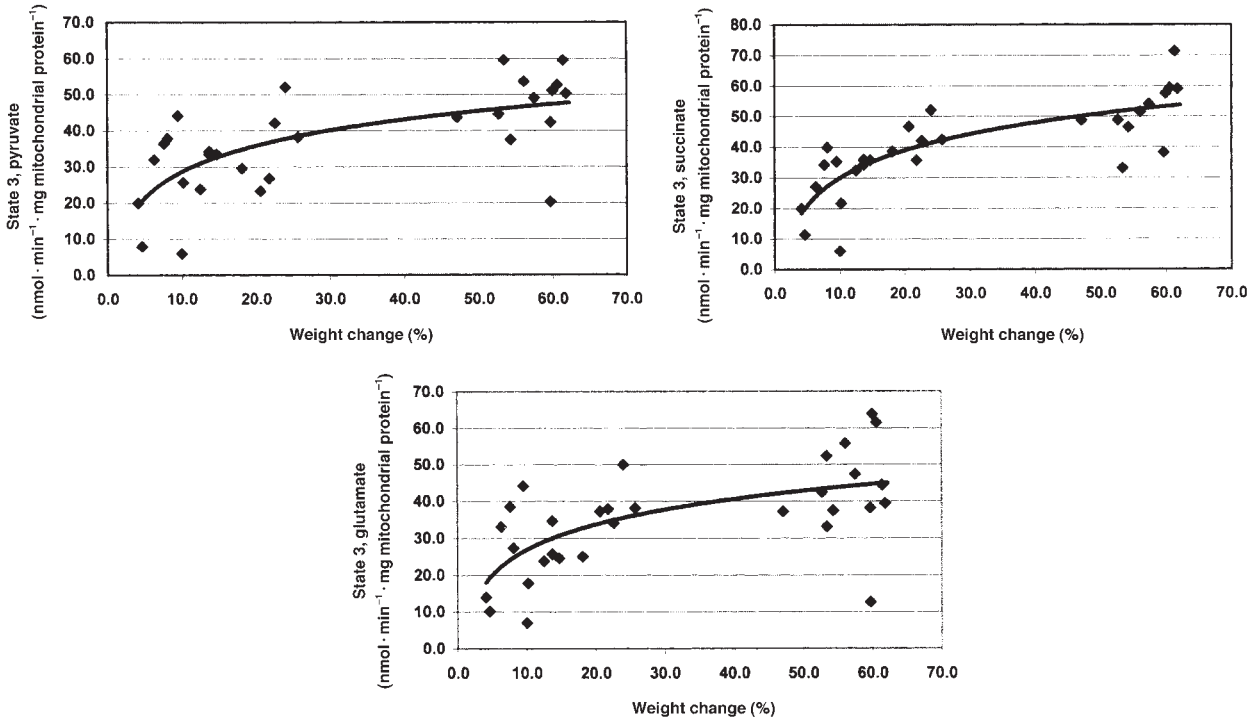


**FIGURE 2.** Relation of state 3 respiration with percentage weight change in control-fed and hypoenergetically fed rats from the start of the diets. The values were normalized to a positive number by adding 40 so as to fit a logarithmic curve. Percentage weight change was significantly correlated with respiration with the use of pyruvate + malate ($r = 0.67$; df = 1, 26; $P < 0.01$), succinate + rotenone ($r = 0.83$; df = 1, 25; $P < 0.01$), and glutamate + malate ($r = 0.74$; df = 1, 27; $P < 0.01$).

## DISCUSSION

Ardawi et al (5) showed that electron transfer in the mitochondria from the gastrocnemius muscle of hypoenergetic rats was low. However, the hypoenergetic rats were fed a diet that had 25% of the nutrients given to controls and received no supplementation with electrolyte, vitamins, and trace elements. Therefore, it was not clear whether the changes were due to energy malnutrition or to deficiencies of electrolytes, vitamins, or trace elements. In addition, Ardawi et al did not study the site of reduced transfer. Furthermore, they did not examine whether there was a reduction in the mitochondrial complex enzymes, which could potentially result in reduced electron transfer. Many different studies have examined the effect of food restriction on mitochondrial function. In obesity, reduced energy intakes did not alter the expression of uncoupling protein messenger RNA in muscle (18). Choline deficiency was shown to alter complex I function (19). In both young and old mice, food restriction reduced the activities of complexes I, III, and IV but did not reduce the activity of complex II (20). In that study (20), the reduced complex activities were associated with a 40% restriction of all nutrients and not specifically of energy. Energy restriction alone with normal protein, mineral, and micronutrient intakes was shown to reduce proton leak (21), but, in the present study, complex activity and its relation to respiratory activity through the different paths were not reported.

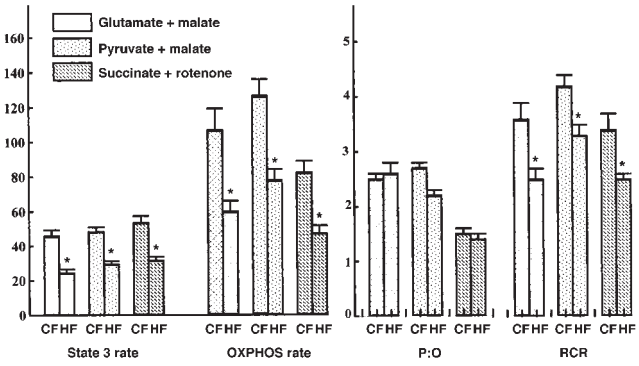


**FIGURE 3.** Mean (±SEM) state 3 respiration (nmol · min$^{-1}$ · mg mitochondrial protein$^{-1}$), oxidative phosphorylation (OXPHOS: nmol ATP · min$^{-1}$ · mg mitochondrial protein$^{-1}$), P:O (mol ATP phosphorous generated/mol oxygen consumed), and the ratio of state 3 to state 4 respiration (respiratory control ratio, RCR) in control-fed (CF) and hypoenergetically fed (HF) rats after 10 d of the diets. State 3 respiration was measured with the use of glutamate + malate ($n = 18$), pyruvate + malate ($n = 17$), and succinate + rotenone ($n = 17$). *Significantly different from CF, $P < 0.002$.

### Quality of mitochondrial preparations from the CF and HF rats

To ensure that the differences between the CF and the HF rats were due to specific changes in electron transfer, it was shown that the mitochondria were impermeable to NADH and that the P:O was not significantly different between the CF and HF rats. In addition, mitochondrial respiration measured on isolation and 2 h later showed that the respiratory characteristics were stable over that period, during which all experiments were completed (data not given). Although the theoretical P:O for NADH substrates is 3 and for FADH substrates is 2, it was recently shown that the

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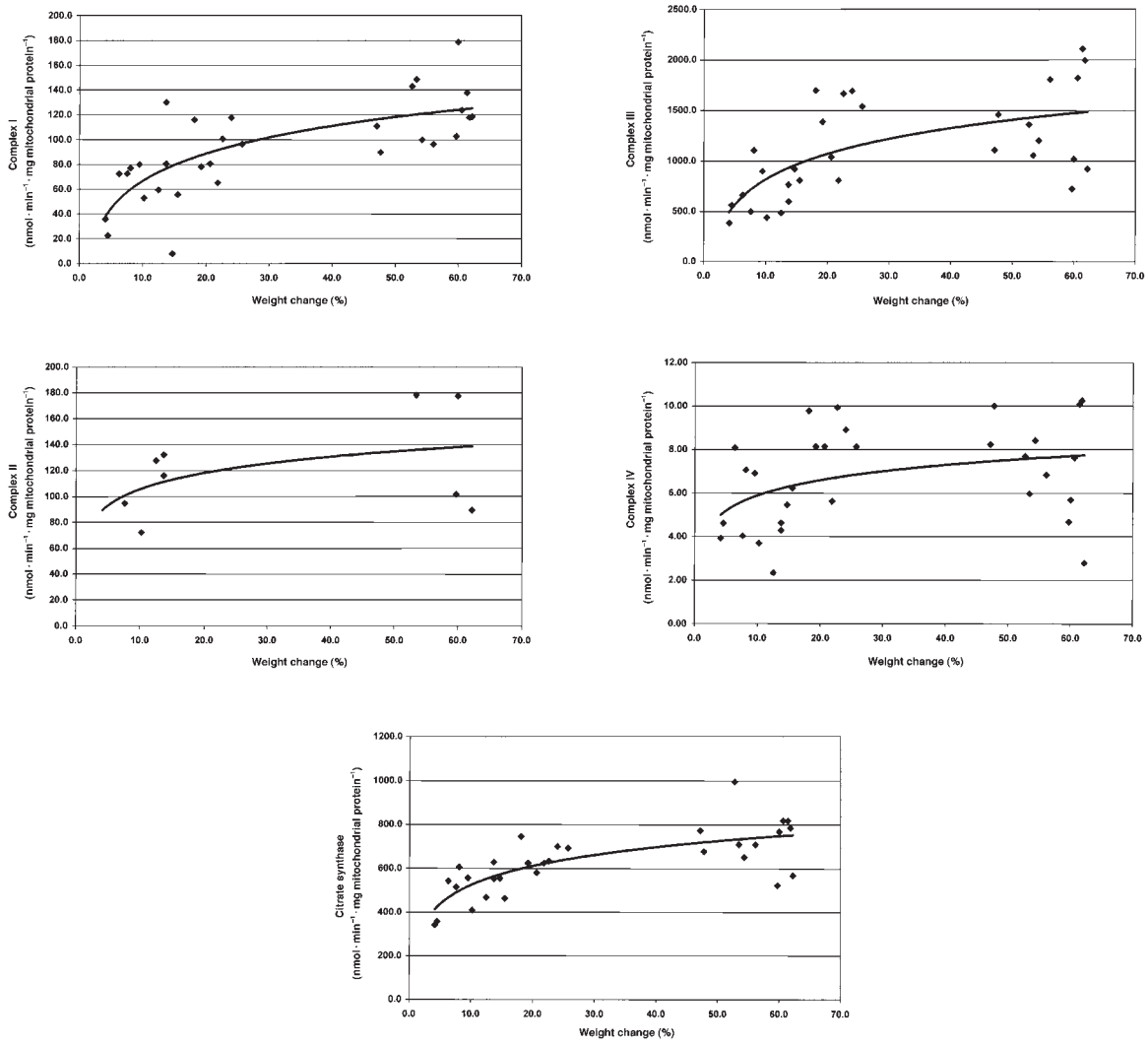


**FIGURE 4.** Relation of the activities of complexes I, II, III, and IV and of citrate synthase with percentage weight change in control-fed and hypoenergetically fed rats from the start of the diets. The values were normalized to a positive number by adding 40 so as to fit a logarithmic curve. Percentage weight change was significantly correlated with complex I ($r = 0.73$; df = 1, 30; $P < 0.01$), complex III ($r = 0.63$; df = 1, 30; $P < 0.01$), and citrate synthase ($r = 0.74$; df = 1, 30; $P < 0.01$).

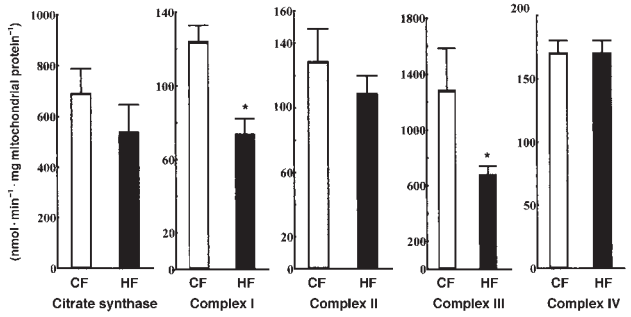


**FIGURE 5.** Mean (±SEM) activities of citrate synthase ($n = 20$) and of complex I ($n = 20$), complex II ($n = 9$), complex III ($n = 20$), and complex IV ($n = 20$) in control-fed (CF) and hypoenergetically fed (HF) rats after 10 d of the diets. *Significantly different from CF, $P < 0.01$.

actual values are different and Hinkle et al (22) obtained ratios closer to 2.5 and 1.5, which are similar to our data (Figure 3). Therefore, the coupling of oxidation to ATP generation of the mitochondria from both the CF and HF rats were good and equivalent to each other.

Furthermore, the similar P:O values found in the CF and HF rats suggest that the physical and chemical pathways of electron transfer are not altered in malnutrition because the same amount of ATP is produced per atom of oxygen consumed in both the control and hypoenergetic conditions. However, the rate of oxygen uptake during ATP production cannot be inferred from these findings because the rate is indicated by the state 3 respiration and the RCR.

Our results showed a significant weight loss–dependent decrease in state 3 respiration with the glutamate, pyruvate, and succinate substrates and a decrease in state 4 respiration with pyruvate + malate as a substrate. These results emphasize that the

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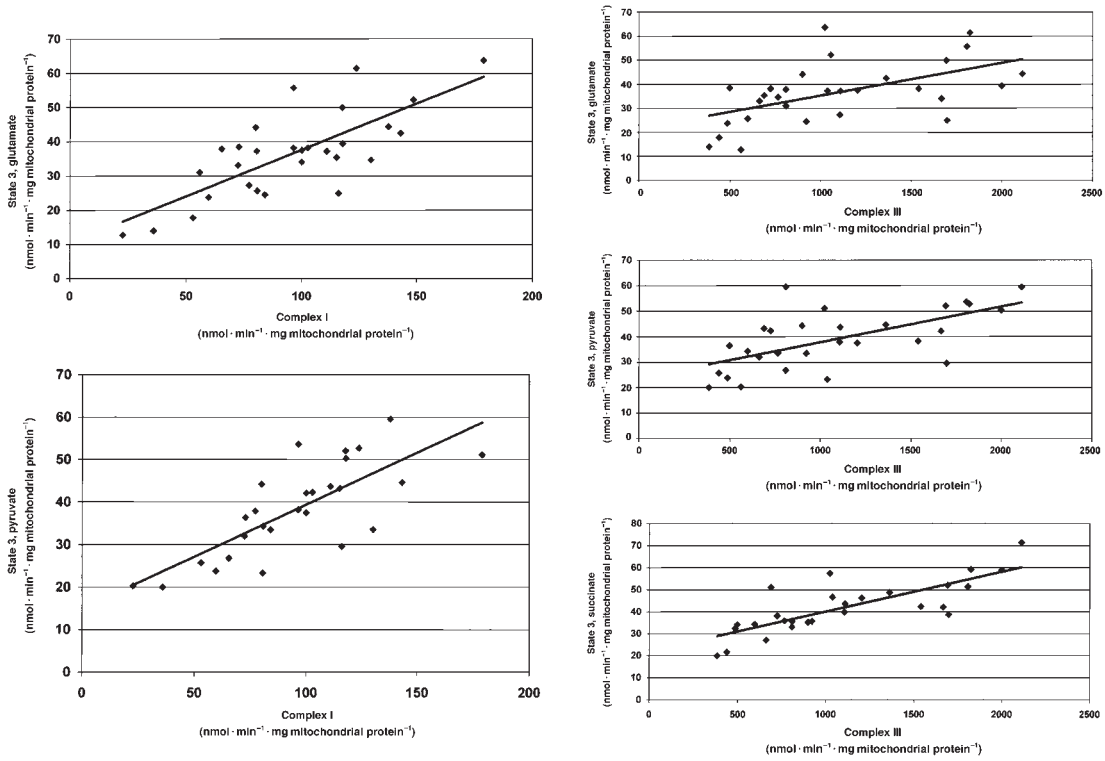


**FIGURE 6.** Correlation between state 3 respiration and complex activities. The correlations between complex I and state 3 respiration with glutamate + malate ($r = 0.755$; df = 1, 27; $P < 0.001$) and pyruvate + malate ($r = 0.76$; df = 1, 25; $P < 0.001$) were significant. The correlations between complex III and state 3 respiration with glutamate + malate ($r = 0.54$; df = 1, 27; $P < 0.005$), pyruvate + malate ($r = 0.63$; df = 1, 26; $P < 0.001$), and succinate + rotenone ($r = 0.78$; df = 1, 25; $P < 0.001$) were significant.

purpose of this investigation was to identify the "weak link" in the electron transport chain that compromises oxidative phosphorylation in malnutrition.

### Effect of nutritional manipulations on oxidative phosphorylation in the gastrocnemius muscle

Our study was the first to show that protein-energy restriction, which is not confounded by micronutrient or electrolyte deficiency, reduces state 3 respiratory rate and oxidative phosphorylation in the gastrocnemius muscle in proportion to the weight loss (Figure 2). The state 4 rate was also reduced significantly only for pyruvate + malate, but the same trend applied to the other 2 substrates: glutamate + malate and succinate + rotenone. The state 3 oxidative phosphorylation was reduced in proportion to the weight loss for 3 different substrates: glutamate, pyruvate, and succinate + rotenone. In contrast, there was little effect of nutrition on the oxidation of TMPD + ascorbate + antimycin A. Therefore, our results support that energy restriction inhibits electron transfer even in the unstimulated mitochondria for complexes I, II, and III after ADP stimulation by complexes I, II, and III or by complexes II and III, with little effect on complex IV and F0F1 ATPase. In addition, the activities of complexes I and III are correlated with weight loss, but those of complexes II and IV are not. However, the relation of complex II may not be significant because of the small sample size. These observations, taken together with the respiratory data, suggest that energy malnutrition probably limits mitochondrial respiration, mainly through the activities of complexes I and III. However, further experiments need to be done to confirm this possibility. Pyruvate and succinate enter the mitochondria through a permease, and glutamate enters via the aspartate-glutamate shuttle. Because their oxidation is inhibited to an equal extent, it is unlikely that limitations in substrate entry explain the reduced oxidative phosphorylation observed in the HF rats. Similarly, because these substrates enter the tricarboxylic acid cycle at different stages in the cycle, it is also unlikely that the generation of NADH or FADH can be responsible for the reduced oxidative phosphorylation observed in the HF rats. Our data do not negate the possibility that other factors known to control mitochondrial respiration—such as proton leak (21), ADP translocator, phosphate carrier, and calcium (23)—may also be influenced by energy malnutrition.

### Effect of nutritional manipulations on the activities of complexes I, III, and IV in the gastrocnemius muscle

Assessment of mitochondrial complex activities showed a significant decrease in enzymes of complexes I and III, with no significant change in complex IV and possibly complex II with weight loss. In addition, there was a significant correlation between the activities of complexes I and III and state 3 respiration, suggesting that a reduction in the activities of complexes I and III plays a role in the observed reduced oxidative phosphorylation rate. Therefore, the decrease in the oxidative phosphorylation activities of complexes II, III, and IV (oxidation with succinate as substrate) may have been due to a decrease in the activity of complex III or of both complexes II and III. However, the decrease in the oxidative phosphorylation activities of

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complexes I, III, and IV (oxidation with glutamate and pyruvate as substrates) may have been due to a combined effect of decreased activities of complexes I and III. These effects, however, may not be additive because the decreased oxidative phosphorylation activities with glutamate and pyruvate compared with succinate are comparable. Conversely, the decrease in complex III enzyme activity may be the rate-limiting reaction, and because both complexes I and II channel electrons into complex III, the pathways for both complexes I and III and for complexes II and III will be affected similarly.

### Effect of nutritional changes on in vitro mitochondrial activity on in vivo energy production

The current study was primarily designed to observe the effect of energy restriction on in vitro oxidative phosphorylation and mitochondrial complex activity in rat muscle. The data showed that a reduction in complex activity was correlated with changes in oxidative phosphorylation. Previously, we showed that the changes in complex I activity of muscle from energy restriction were correlated with similar changes in complex I of lymphocytes (6). Furthermore, nutritional manipulations had the same effects on human lymphocytes (7, 8); therefore, it is likely that the effect of dietary energy restriction seen in rats is applicable to humans.

The influence of reduced oxidative phosphorylation in isolated mitochondria on energy production in vivo will depend on the effect of energy restriction on mitochondrial and fiber distribution, on the ratio of activity to muscle protein, and on whether the reduction in maximal in vitro activity actually reduces the ability of muscle to produce energy in vivo. Although these factors were not examined in the current study (24), in another study, in which energy-restricted obese humans were fed a diet providing only 2000 kJ/d (≈500 kcal/d), the authors concluded that "no changes occurred in fiber type distribution or activities of enzymes reflecting β-oxidation and mitochondrial density." On the basis of that study, which showed that confounding factors such as fiber distribution and mitochondrial density do not seem to be altered by a low-energy diet (24), it is likely that the reduction in in vitro mitochondrial oxidative phosphorylation may limit muscle oxidative phosphorylation in vivo. It should be recognized that because this study in humans was conducted in obese subjects, the effect of energy restriction on fiber type and muscle density may not apply to malnourished subjects. The next question to be answered is whether the reduced capacity of in vitro oxidative phosphorylation limits energy production in vivo. Again, in the abovementioned study (24), dietary energy restriction reduced exercise-induced energy expenditure. Because the increase in oxygen consumption with exercise is mainly in muscle, the consumption of an energy-restricted diet—even by obese subjects—influences muscle oxygen consumption.

However, it will be necessary to conduct additional studies in which mitochondrial oxidation and its effect in vivo are manipulated to be certain that the in vitro data described here can be translated to in vivo changes in energy production.

### Conclusion

Our study showed that the complex activities of the mitochondrial oxidative phosphorylation chain (oxidative phosphorylation system) in muscle were depressed selectively with energy deprivation when compared with normally fed rats. These findings may partly explain the mechanism of reduced muscle energetics in energy malnutrition.

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