Nutritional effects on mitochondrial bioenergetics

Alterations in oxidative phosphorylation by rat liver mitochondria

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Rats malnourished since birth and fed on a protein-free diet for 2 weeks showed a 23–27% decrease in the State-3 oxidation of glutamate, succinate and ascorbate + *NNN*'-tetramethyl-*p*-phenylenediamine by liver mitochondria compared with control fed animals. ATP synthesis and the respiratory control index were diminished at the three coupling sites, but significant alterations were not observed in ADP/O ratios. $V_{\max}$ for NADH oxidation in electron-transport particles was 40% lower. Mitochondrial cytochromes *b* and *c*₁ remained unchanged, but cytochrome *c* was increased by 26%. Cytochromes *a* + *a*₃ were diminished by 22%. $V_{\max}$ for mitochondrial ATPase was 23% lower. These results suggest that the lower content of cytochrome *a* + *a*₃ at the rate-controlling step of oxidative phosphorylation in malnourished rats might be mainly responsible for the decrease in substrate oxidations as well as ATP synthesis at the three coupling sites. The decreased synthesis and hydrolysis of ATP suggests that other energy-dependent mitochondrial processes could be decreased during malnutrition.

The nutritional status of rats affects bioenergetic function. Studies of alterations in mitochondrial activity by nutritional status have been oriented mainly to the effects of starvation, short periods of starvation or deficiencies in specific nutrients, but very little is known about the effects of prolonged drastic malnutrition. The oxidative activity of rat liver mitochondria towards substrates which activate different segments of the respiratory chain were decreased drastically during starvation (Rasheed *et al.*, 1980). Casciaro *et al.* (1978) observed changes in energy metabolism during prolonged starvation, reporting decreased body weight, respiration, mitochondrial protein and cytochrome oxidase and succinate dehydrogenase activities.

The composition of the diet plays an important role in mitochondrial oxidative phosphorylation. Rats chronically fed on a diet containing long-chain fatty acids showed a decline in the oxidation of substrates at coupling sites I and II as well as a

decrease in ATP synthesis in heart mitochondria (Clandinin, 1978). Deficiency of vitamin E resulted in a respiratory decline in rat liver mitochondria (Schwarz, 1972; Grove *et al.*, 1965), but adequate dietary vitamin C supplementation protected normal respiration and swelling rates of liver mitochondria when vitamin E was deficient (Chen & Chen, 1978).

Studies on rat skeletal muscle have shown that dietary iron deprivation decreases mitochondrial oxidative activities without affecting phosphorylative efficiency (Finch *et al.*, 1976). However, the content of Fe-S proteins and dehydrogenase activities were decreased (Maguire *et al.*, 1982).

The studies described in the present paper report the effect of protein-energy malnutrition on oxidative phosphorylation in rat liver mitochondria. We demonstrate that oxidation of substrates, the phosphorylation of ADP and the respiratory control index are significantly lower at the three coupling sites in mitochondria from malnourished rats, but the ADP/O ratio remained unaffected. Mitochondria from malnourished rats also showed a lower uncoupler-elicited ATPase activity. These results suggest that malnutrition affects oxidative phosphorylation as well as ATP hydrolysis.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; TMPD; *NNN*'-tetramethyl-*p*-phenylenediamine.

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Materials and methods

Animals and diets

Two groups of male Wistar rats, 35 days old, were used in all experiments. (a) Malnourished group. Protein-energy malnutrition was provoked as follows: after birth, 16 offspring from the same litter were nursed by one mother for 21 days, then fed for 14 days *ad libitum* with water and a protein-free diet as described by Salazar *et al.* (1983). (b) Normal group. After parturition, eight offspring from the same litter were nursed by one mother for 21 days, then fed for 14 days *ad libitum* with water and a commercial balanced pellet diet purchased from FABALIM, Chile.

Preparation of mitochondria

Mitochondria from 35-day-old male rats of both groups were isolated by a standard procedure of our laboratory as described by Gil *et al.* (1980). The homogenization medium was 0.25M-sucrose/0.5mM-EGTA (pH 7.2)/0.1% bovine serum albumin (crystallized and freeze-dried). The mitochondrial pellet was washed three times with 0.25M-sucrose, pH 7.2. The final mitochondrial pellet was suspended in 0.25M-sucrose to give a concentration of 50 mg of protein/ml.

Determination of O₂ consumption and H⁺ movements

O₂ consumption was measured polarographically with a Clark electrode no. 5331 (Yellow Springs Instrument Co.) by using a YSI model 53 O₂ monitor linked to a 100mV mono-channel Goerz RE 511 recorder. H⁺ movements were followed with a combined glass pH-electrode (Thomas 4094 L25), with an Orion model 901 digital pH-meter linked to another 10mV mono-channel Goerz RE 511 recorder. The absolute amounts of H⁺ uptake were determined by back-titration with known amounts of 0.1M-HCl. The 2.0ml reaction medium consisted of: 117mM-LiCl, 13mM-KCl, 3.0mM-Hepes, 2.0mM-potassium phosphate, pH 7.2, and 2.5–5.0mg of mitochondrial protein (Reynafarje *et al.*, 1976). Substrate concentrations were 2.0mM-glutamate, or 2.5mM-succinate, or 5.0mM-ascorbate + 0.5mM-TMPD; other additions were 0.2–0.4mM-ADP, 4μM-rotenone and 0.4μM-antimycin A. The system was equilibrated with mitochondria at 25°C for 2min; then the rates of O₂ consumption and H⁺ uptake were determined. Data recorded represent the averages of five to nine experiments. All determinations were made without undue lapse of time after isolation of mitochondria.

Electron-transport particles

These were obtained by ultrasonic disruption of liver mitochondria from normal fed and malnourished rats by a modification of the method of

Ruzicka & Crane (1970). Mitochondria were suspended in 0.25M-sucrose (pH 7.2) at a protein concentration of 30mg/ml. Sonication was accomplished with a Braun-Sonic 1510 sonifier with an intermediate titanium probe (length 127mm, diameter 9.5mm) set at 200W. To establish better sonication conditions, a curve of NADH oxidase activity versus sonication time was performed for both types of electron-transport particles. The data indicated that higher NADH oxidase activity was obtained with seven 1 min intervals for mitochondria from normal rats and five 1 min intervals for mitochondria from malnourished rats. All sonications were performed in a vessel submerged in ice/NaCl solution. The supernatant obtained by centrifugation at 26000g for 15min was re-centrifuged at 105000g for 45min. The pellet (electron-transport particles) was washed twice with 0.25M-sucrose, pH 7.2, by homogenization with a glass homogenizer fitted with a Teflon pestle, followed by centrifugation at 105000g for 45min. The electron-transport particles were resuspended in 0.25M-sucrose, pH 7.2, at a concentration of 15–20mg of protein/ml and stored at –20°C.

Determination of NADH oxidase activity

This was done by a modification of the method of Singer (1974). The assay system contained, in 3ml: 100mM-K₂HPO₄, pH 7.4, 0.032mM-cytochrome c, 0.0075–0.25mM-NADH and electron-transport particles (0.12mg of protein). The decrease in A₃₄₀ was measured at 25°C in a Shimadzu UV-210 A double-beam spectrophotometer.

ATPase activity

ATPase activity (EC 3.6.1.3) was measured in liver mitochondria from normal and malnourished rats. The reaction medium contained, in 2.0ml: 117mM-LiCl, 13mM-KCl, 10mM-Hepes, pH 7.4, and 2mg of mitochondrial protein. The system was preincubated for 3min at 25°C; then 3mM-ATP and 0.1–1.0μM-CCCP were added simultaneously. Hydrolysis was allowed to proceed for 3min. To stop the reaction, trichloroacetic acid (final concn. 10%, w/v) was added. After centrifugation at 12000g for 1min, P_i was determined in the supernatant essentially as described by Baginski *et al.* (1967).

Determination of cytochromes

Cytochromes *a* + *a*₃, *b*, *c*₁ and *c* were measured at 25°C with mitochondria (5mg of protein) treated with cholate/phosphate (Williams, 1964), in a Shimadzu UV-210 double-beam spectrophotometer by using the dithionite-reduced sample versus the oxidized reference, and the concentrations were calculated by the method of Williams (1964).

Assay of total protein

Protein was determined in samples of mitochondria by the method of Murphy & Kies (1960), standardized with bovine serum albumin.

Statistical analysis

The experimental data in the Tables were analysed by Student's *t* test. The numbers of separate observations (*n*) and the standard deviations of the mean are given in the respective Tables. The data in the Figures were fitted by linear regression (Steel & Torrie, 1960).

Chemicals

All chemicals used were of the purest grade available and were obtained from Sigma Chemical Co., Nutritional Biochemical Corp. or Merck.

Results

Body and liver weight, food intake and mitochondrial protein

The model of protein-energy malnutrition used in this study provoked drastic changes in body and liver weight and in mitochondrial protein content in 35-day-old male rats. Table 1 shows that body and liver weights are decreased to 20% or less of normal in the malnourished animals. On the other hand mitochondrial protein content was 27% lower in the malnourished group.

The average body weights of the two groups at the end of weaning but before diet administration were 35.5 ± 2.18 g (normal) and 25.0 ± 2.20 g (malnourished). The liver weights were 1.5 ± 0.12 g and 0.82 ± 0.07 g for normal and malnourished rats respectively.

Oxidative activities

The rates of O_2 consumption and H^+ uptake during oxidation of common substrates which

activate different segments of the respiratory chain were measured in the presence and absence of ADP in liver mitochondria isolated from normal and malnourished rats. Data for oxidation of glutamate, succinate and ascorbate + TMPD are given in Table 2. In State 3, the rate of O_2 uptake decreased by 23% for oxidation of glutamate, 27% for succinate and 26% for ascorbate plus TMPD. Similarly, the rate of H^+ uptake was also lower during the oxidation of the three substrates by malnourished mitochondria; the decreases were respectively 31%, 38% and 30% for oxidation of glutamate, succinate and ascorbate plus TMPD. In the absence of ADP (State 4), O_2 consumption was diminished by about 18% during oxidation of succinate and ascorbate + TMPD, but remained unchanged during oxidation of glutamate.

Table 1. Effect of protein-energy malnutrition on the body weight, liver wet weight, food intake and liver mitochondrial protein

All determinations were made in 35-day-old male rats. Malnutrition was provoked and liver mitochondria from normal and malnourished rats were isolated as described in the Materials and methods section. Mitochondrial protein was measured by the method of Murphy & Kies (1960). The values are the means $\pm$ S.D. for 29 (normal) and 19 (malnourished) independent determinations on groups of rats (normal group, two or three rats; malnourished group, four to nine rats). Significance (normal versus malnourished): * $P < 0.01$; ** $P < 0.001$.

	Normal	Malnourished
Body wt. (g)	101.9 ± 8.6	$20.0 \pm 1.9^{**}$
Liver wet wt. (g)	4.96 ± 0.82	$0.77 \pm 0.09^{**}$
Food intake (kcal/day)	21.48 ± 2.20	$9.10 \pm 0.58^{**}$
(kJ/day)	89.93 ± 9.21	$38.10 \pm 2.43^{**}$
Mitochondrial protein (mg/g of liver)	30.5 ± 7.2	$22.3 \pm 4.9^*$

Table 2. Rates of oxidation of glutamate, succinate and ascorbate + TMPD by rat liver mitochondria

O_2 and H^+ uptakes were measured as described in the Materials and methods section, and are expressed as ng-atoms of O /min per mg of protein and as ng-ions of H^+ /min per mg of protein respectively. Determinations of the rate of State-3 oxidations were measured after the addition of 400 nmol of ADP for oxidation of glutamate and succinate and of 200 nmol of ADP for oxidation of ascorbate + TMPD. The mitochondrial protein concentration was 2.5 mg/ml for oxidation of glutamate and succinate and 1.25 mg/ml for oxidation of ascorbate + TMPD. The following inhibitors were used: 4 μ M-rotenone for succinate oxidation; 4 μ M-rotenone and 0.4 μ M-antimycin A for oxidation of ascorbate + TMPD. The rates of State-4 oxidations were determined after ADP exhaustion. The values are means $\pm$ S.D. for six (normal) and seven (malnourished) independent determinations on groups of rats. Significance (normal versus malnourished): * $P < 0.025$; ** $P < 0.01$.

Substrate	State 3 O_2 uptake		State 3 H^+ uptake		State 4 O_2 uptake	
	Normal	Malnourished	Normal	Malnourished	Normal	Malnourished
Glutamate	59.7 ± 6.8	$46.1 \pm 3.7^{**}$	119.8 ± 23.6	$83.0 \pm 14.6^*$	9.8 ± 0.6	10.8 ± 1.4
Succinate	134.2 ± 13.5	$98.1 \pm 10.7^*$	184.8 ± 26.3	$115.3 \pm 21.6^*$	31.0 ± 5.4	$24.8 \pm 1.3^*$
Ascorbate + TMPD	167.2 ± 29.6	$124.8 \pm 6.0^*$	321.6 ± 69.6	$226.5 \pm 26.0^*$	109.2 ± 17.3	$88.8 \pm 4.7^*$

Table 3. *Respiratory control index, ADP/O ratio and ATP synthesis*

Respiratory control indices and ADP/O ratios were determined as described by Chance & Williams (1956). ATP synthesis is expressed as nmol of ATP/min per mg of mitochondrial protein, and it was calculated by assuming total conversion of ADP into ATP at the corresponding experimental ADP/O ratio. The values are means $\pm$ S.D. for six (normal) and seven (malnourished) independent determinations on groups of rats. Significance (normal versus malnourished): * $P < 0.05$; ** $P < 0.025$.

Substrate	Respiratory control index		ADP/O ratio		ATP synthesis	
	Normal	Malnourished	Normal	Malnourished	Normal	Malnourished
Glutamate	6.11 ± 0.41	$4.33 \pm 0.74^{**}$	2.75 ± 0.13	2.69 ± 0.23	165.0 ± 26.5	$125.0 \pm 20.5^{**}$
Succinate	4.70 ± 0.25	$3.97 \pm 0.57^{**}$	1.66 ± 0.09	1.68 ± 0.14	224.0 ± 34.5	$166.3 \pm 31.6^{**}$
Ascorbate + TMPD	1.52 ± 0.10	1.41 ± 0.12	0.98 ± 0.11	0.98 ± 0.10	167.1 ± 47.4	$122.9 \pm 18.3^*$

At the end of weaning, O_2 consumption in State 3 for glutamate oxidation was similar in both groups, 64.6 and 64.4 ng-atoms of O /min per mg of protein. Likewise, O_2 consumption for succinate oxidation was 114.8 and 110.8 ng-atoms of O /min per mg of protein. These results clearly showed that alterations in oxidative activities are not observed during weaning.

ATP synthesis, respiratory control index and ADP/O ratio

A 25% decline was observed for ATP synthesis at the three coupling sites in mitochondria from malnourished rats, as shown in Table 3. In Table 3 data are also given for the respiratory control index measured during oxidation of glutamate, succinate and ascorbate + TMPD. Interestingly, the respiratory control index was lower in mitochondria from malnourished rats as compared with normal fed rats, but no significant alterations in ADP/O ratio were observed at the three coupling sites.

NADH oxidase

Respiratory-chain activity from NADH to O_2 was determined in electron-transport particles obtained by ultrasonic disruption of liver mitochondria from normal fed and malnourished rats, as shown in Fig. 1. The K_m values for NADH oxidation were the same in normal and malnourished rats (15 μM), but V_{max} values were significantly different; 1.29 for electron-transport particles from normal rats and 0.78 for those from malnourished rats, a difference of about 40%.

Cytochrome content

Respiratory cytochromes were assayed in liver mitochondria from normal and malnourished rats. The values given in Table 4 showed that the contents of cytochromes b , c_1 and c were higher in mitochondria from malnourished rats, although the values for cytochromes b and c_1 were not significantly so ($P < 0.5$). On the other hand,

Fig. 1. *Effect of varying the concentration of NADH on the NADH-oxidative activity of electron-transport particles: Lineweaver-Burk plot*

Electron-transport particles obtained from normal (●) and malnourished (Δ) rat liver mitochondria were incubated as described in the Materials and methods section. The values are means $\pm$ S.D. for five (normal) and six (malnourished) independent determinations. Linear regression: $r = 0.9992$ (normal); $r = 0.9987$ (malnourished). V_{max} : 1.29 ± 0.07 (normal); 0.78 ± 0.04 (malnourished).

cytochromes $a + a_3$ were decreased by 22% in mitochondria from malnourished rats ($P < 0.025$).

ATPase activity

In order to compare ATPase activities in intact mitochondria from both groups, it was necessary to choose experimental conditions to maximize the ATPase activity; this was obtained at saturating ATP concentration (3 mM). In that condition the V_{max} values for ATPase were dependent on uncoupler concentration.

V_{max} and K_a for ATPase of mitochondrial suspensions from normal and malnourished rats were estimated from double-reciprocal plots of

Table 4. *Cytochrome contents of rat liver mitochondria*

The cytochrome contents of freshly prepared hepatic mitochondria were determined as described by Williams (1964). Other details are given in the Materials and methods section. The values are means $\pm$ S.D. for five independent determinations on groups of rats (normal or malnourished). Significance (normal versus malnourished): * $P < 0.025$; ** $P < 0.01$; other changes, $P < 0.5$.

	Cytochrome content		
	(nmol/mg of protein)		(% change)
	Normal	Malnourished	
Cytochromes $a + a_3$	0.224 ± 0.024	$0.176 \pm 0.015^*$	-21.6
Cytochrome b	0.144 ± 0.026	0.164 ± 0.024	+12.2
Cytochrome c_1	0.088 ± 0.009	0.110 ± 0.033	+20.0
Cytochrome c	0.173 ± 0.016	$0.235 \pm 0.035^{**}$	+26.4

Fig. 2. Effect of varying the concentration of CCCP on the ATPase activity of rat liver mitochondria: Lineweaver-Burk plot

Mitochondria obtained from normal (●) and malnourished (△) rat liver were incubated as described in the Materials and methods section. The values are means $\pm$ S.D. for eight (normal) and nine (malnourished) independent determinations. Linear regression: $r = 0.9985$ (normal); $r = 0.9997$ (malnourished). $V_{\max}$: 0.725 ± 0.020 (normal); 0.561 ± 0.062 (malnourished). K_a : $0.285 \pm 0.019 \mu\text{M}$ (normal); $0.164 \pm 0.059 \mu\text{M}$ (malnourished).

initial velocities versus CCCP concentrations, as shown in Fig. 2. $V_{\max}$ was decreased by 23% in malnourished rats, and K_a values (conc. of CCCP required for inducing half-maximal activity) for CCCP were $0.285 \mu\text{M}$ in mitochondria from normal rats and $0.164 \mu\text{M}$ for malnourished rats.

ADP-ATP transport system

The time course of ADP uptake showed that the nucleotide uptakes in normal and malnourished mitochondria were similar and that the nucleotide uptake was essentially completed in less than 30 s in both types of mitochondria (results not shown).

Discussion

Drastic alterations in liver microsomal oxidative activities have been reported with the same experimental model of protein-energy malnutrition as used in the present work (Salazar *et al.*, 1983). Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity was almost negligible in malnourished rats, in contrast with the high values observed in normal fed rats. However, re-feeding malnourished rats with a protein diet enhanced the hydroxylase activity to values even higher than those observed in controls. These changes in microsomal oxidative activities were correlated with nutritionally provoked alterations in cytochrome P-450 species involved on benzo[*a*]pyrene oxidation.

In the present study we report that a protein-energy deficiency is associated with alterations in liver mitochondrial bioenergetic functions. The expected drastic decrease in body and liver weights observed in malnourished animals was accompanied by a significant decrease in mitochondrial protein.

The oxidative activity of the respiratory chain was significantly diminished in mitochondria from malnourished rats, since the rates of oxidation of substrates in the presence of ADP at the three coupling sites were always lower in malnourished rats. Interestingly, the percentage decreases in activities were very similar for the three substrates assayed (range 23–27%). On the other hand, the $V_{\max}$ for NADH oxidation measured in electron-transport particles showed a 40% decline in mitochondria from malnourished rats. These results, as well as the lower rate of oxidative activity of malnourished mitochondria in the absence of ADP, suggested alterations in the respiratory chain. Erecinska & Wilson (1978) consider that the rate-controlling step of oxidative phosphorylation is located at cytochrome oxidase. Bohnensack *et al.* (1982) confirmed the finding of Erecinska &

Wilson (1978), but suggest that other factors control oxidative phosphorylation, i.e. the supply of substrates and adenine nucleotide translocation. We have not measured all the components of the electron-transport chain, but we have found changes in the amounts of some cytochromes. Cytochrome *c* in malnourished rats appears to be increased by up to 26%, whereas cytochromes *a* + *a*₃ are significantly diminished (22%), with respect to the values observed in normal fed rats. The lower contents of cytochromes *a* + *a*₃ could account for most of the decreased rate of oxidative activities at the three coupling sites. However, we cannot discard the possibility that changes occur in the contents of other components, such as flavoproteins and phospholipids, which could also affect mitochondrial oxidative activities. It seems reasonable that, in a very drastic state of malnutrition, the organism makes an effort to regulate the energy process by affecting the rate-controlling step of oxidative phosphorylation, i.e. cytochrome oxidase. We have no experimental basis to explain the lower contents of cytochromes *a* + *a*₃ in mitochondria from malnourished rats, but a possible explanation could be that the lack of protein in the diet for a prolonged period of time could affect the rate of synthesis of these cytochromes or their assembly in the membrane, and/or could enhance the degradation of cytochromes *a* + *a*₃ previously assembled in the mitochondrial membrane.

As expected, an alteration in the respiratory chain affected the rate of phosphorylation. Therefore mitochondria from malnourished rats showed a lower rate of ATP synthesis at the three coupling sites.

The respiratory control index measured in mitochondria from normal fed and malnourished rats showed that the lower rate of ATP synthesis was not due to uncoupling in malnourished rats. However, the decrease on the respiratory control index observed in mitochondria from malnourished rats at the three coupling sites may be the results of the lower rates of oxidation measured in State 3.

It is noteworthy that, even though maximal rates of substrate oxidation were suppressed by 23–27% at the three coupling sites in mitochondria from malnourished rats, there was no loss in the efficiency of conversion of ADP into ATP, since the ADP/O ratio remained unchanged. It is clear that liver mitochondria from malnourished rats readily perform their major function of ATP synthesis, albeit at a lower rate than mitochondria from normal fed rats. This indicates that the mitochondrial electron transport functioned normally. It follows that the sequence of electron flow for energy coupling had not been altered, as

this would presumably decrease the efficiency of ATP synthesis, but instead there was a decrease in a functional electron-carrying component at the rate-controlling step. The remaining functional components were able to maintain an adequate electrical and proton gradient to facilitate ATP synthesis in spite of the poor nutritional condition.

To prevent the increase in the energy-yielding catabolic process over energy-consuming anabolic reactions during protein-energy malnutrition, it is expected that synthesis of ATP, as well as ATP hydrolysis, must be regulated. To verify this assumption, ATP activity was assayed in the presence of the uncoupler CCCP in mitochondria from normal and malnourished rats. The data showed that ATPases from normal fed and malnourished rats were both stimulated by CCCP. The concentration of the uncoupler required for inducing half-maximal activity (*K*_a) was 42% lower in mitochondria from malnourished rats, suggesting a better binding of the uncoupler. Furthermore, the *V*_{max} was 23% lower in mitochondria from malnourished rats.

In conclusion, mitochondria from malnourished rats showed an alteration in the electron-transport chain as well as in the ATPase; however, the ADP-ATP transport system remained unaffected.

In addition to ATP synthesis, ATP hydrolysis is also decreased by malnutrition, and it is expected that many other energy-dependent processes in the mitochondria might be decreased during malnutrition. Studies in our laboratory indicated that the Ca²⁺ uptake by liver mitochondria supported by electrical gradients derived either from electron transport or ATP hydrolysis is diminished drastically in mitochondria from malnourished rats (J. Ferreira & L. Gil, unpublished work). Since mitochondria probably play an important role in regulation of Ca²⁺ concentration in the extramitochondrial milieu (Fiskum & Lehninger, 1980), possibly in bone calcification (Lehninger, 1977), the effects of protein-energy malnutrition on mitochondrial bioenergetics might have important physiological consequences.

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