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Mitochondrial Adaptation to *in vivo* Polyunsaturated Fatty Acid Deficiency: Increase in Phosphorylation Efficiency[1]

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Polyunsaturated fatty acid (PUFA) deficiency affects respiratory rate both in isolated mitochondria and in hepatocytes, an effect that is normally ascribed to major changes in membrane composition causing, in turn, protonophoriclike effects. In this study, we have compared the properties of hepatocytes isolated from PUFA-deficient rats with those from control animals treated with concentrations of the protonophoric uncoupler 2,4-dinitrophenol (DNP). Despite identical respiratory rate and *in situ* mitochondrial membrane potential ($\Delta\Psi$), mitochondrial and cytosolic ATP/ADP–P_i ratios were significantly higher in PUFA-deficient cells than in control cells treated with DNP. We show that PUFA-deficient cells display an increase of phosphorylation efficiency, a higher mitochondrial ATP/ADP–P_i ratio being maintained despite the lower $\Delta\Psi$. This is achieved by (1) decreasing mitochondrial P_i accumulation, (2) increasing ATP synthase activity, and (3) by increasing the flux control coefficient of adenine nucleotide translocation. As a consequence, oxidative phosphorylation efficiency was only slightly affected in PUFA-deficient animals as compared to protonophoric uncoupling (DNP). Thus, the energy waste induced by PUFA deficiency on the processes that generate the proton motive force (pmf) is compensated *in vivo* by powerful adaptive mechanisms that act on the processes that use the pmf to synthesize ATP.

KEY WORDS: Respiratory chain; ATP synthesis; PUFA; uncoupler; hepatocyte; mitochondria; proton motive force.

INTRODUCTION

The mitochondrial lipid composition is known to affect the oxidative phosphorylation efficiency (Brand *et al.*, 1991; Brookes *et al.*, 1997a,b, 1998) as well as some enzyme activities (Dabbeni-Sala *et al.*, 1981; Paradies and Ruggiero, 1991; Streicher-Scott *et al.*, 1994). Changes in phospholipid content may be involved in pathological situations affecting the yield of oxidative phosphorylation, such as hyper- or hypothyroidism (Brand *et al.*, 1992; Hulbert *et al.*, 1976; Ida Chen and Hoch, 1977; Paradies *et al.*, 1993). Moreover, it could explain the differences observed in diverse animal species under physiological conditions (Brand *et al.*, 1991; Brookes *et al.*, 1998; Porter *et al.*, 1994). Dietary PUFA deficiency is responsible for a large change in membrane phospholipid composition. This change involves the mitochondrial membranes and affects oxidative phosphorylation (Christensen, 1986; Deaver *et al.*, 1986; Divakaran and Venkataraman, 1977; Fontaine *et al.*, 1996; Piquet *et al.*, 1996; Stancliff *et al.*, 1969), but whether a decreased efficiency of oxidative phosphorylation occurs

[1] Key to abbreviations: PUFA, polyunsaturated fatty acid; DNP, 2,4-dinitrophenol; ANC, adenine nucleotide carrier; $\Delta\Psi$, electrical potential difference across the mitochondrial inner membrane; ΔpH, proton chemical potential difference across the mitochondrial inner membrane; Δp, proton motive force (pmf).

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is still the matter of controversy. Indeed, ATP/O ratios have been reported to be either unaffected (Christensen, 1986; Deaver *et al.*, 1986; Stancliff *et al.*, 1969) or decreased (Divakaran and Venkataraman, 1977; Fontaine *et al.*, 1996). Furthermore, the physiological consequences of PUFA deficiency remain confusing especially because of the striking differences observed between isolated mitochondria and intact cells (Fontaine *et al.*, 1996; Leverve *et al.*, 1998; Piquet *et al.*, 1996; Rigoulet *et al.*, 1998).

Assessing the occurrence of changes of oxidative phosphorylation *in situ* is extremely difficult because the overall efficiency of this pathway can be affected at multiple levels: (1) at the point of coupling between electron and proton fluxes through the respiratory chain (redox slipping); (2) at the level of the proton leaks across the mitochondrial inner membrane; and (3) at the level of coupling between proton flux and ATP synthesis through the ATP synthase. Studies with both isolated mitochondria and intact cells have shown that PUFA deficiency causes an increase in proton permeability of the inner mitochondrial membrane, similar to that caused by the classical protonophore 2,4-dinitrophenol (DNP). Treatment with the latter, indeed, causes an increase of respiratory rate, matching the decrease of membrane potential, as well as to the expected decrease of cytosolic and mitochondrial ATP/ADP ratios (Azzone *et al.*, 1978; Krishnamoorthy and Hinkle, 1984; Nicholls, 1974; Nobes *et al.*, 1990; Sibille *et al.*, 1995).

The present study was undertaken to resolve current discrepancies about the mitochondrial effects of PUFA deficiency and to provide a mechanistic explanation for the changes it induces on the efficiency of oxidative phosphorylation. By comparing the effects of PUFA deficiency with those of matching DNP concentrations in liver cells, we found striking differences in the parameters regulating oxidative phosphorylation. Indeed, for the same respiratory rates and *in situ* mitochondrial membrane potential, both cytosolic and mitochondrial phosphate potentials were significantly higher in cells from PUFA-deficient rats than in DNP-treated control cells. We show that this result was achieved by decreasing mitochondrial P_i accumulation, increasing ATP synthase activity, and by increasing the flux control coefficient of adenine nucleotide translocation. As a consequence, oxidative phosphorylation efficiency is only slightly affected in PUFA-deficient animals in contrast to the drastic effect of protonophoric uncoupling. Thus, the energy waste induced by PUFA deficiency on the processes that generate the pmf is offset *in vivo* by powerful adaptive mechanisms that act on the processes that use the pmf to synthesize ATP.

MATERIALS AND METHODS

Male weanling Wistar rats (21 days old, 50–60 g) were fed a semisynthetic diet containing (% mass): casein 21, maize starch 44.2, sucrose 23.4, cellulose 1.8, D,L-methionine 0.1, mineral mixture 3.3, and vitamin mixture 0.9 for at least 6 weeks. This diet was supplemented with either stearic and palmitic acid (2.65% mass of each, PUFA-deficient diet) or soya oil (5.3% mass, control diet). Animals had ad libitum access to food and water.

Hepatocytes were isolated by the method of Berry and Friend (1969) as modified by Groen *et al.* (1982a), from rats fasted for 20–24 h. Liver cells (10–12 mg dry cells/ml) were incubated as described in Espié *et al.*, (1995) with the amounts of substrates indicated in Table I. After 25 min of incubation, myxothiazol-sensitive oxygen consumption was measured. At the same time, 300 μl of the cell suspension were withdrawn, and mitochondrial and cytosolic contents were separated by using the digitonin fractionation method (Zuurendonk and Tager, 1974). ATP and ADP were measured by HPLC as described previously (Argaud *et al.*, 1993) and P_i was measured as described by Summer (1944). The intramitochondrial $NADH/NAD^+$ ratio was determined by the metabolite indicator method (Akerboom *et al.*, 1979) assuming the 3-hydroxybutyrate dehydrogenase reaction is in nearequilibrium $\{K_{app} = [AcAc] \times [NADH]/[3\text{-OHBu}] \times [NAD^+]$, *i.e.*, 4.93×10^{-2} (Williamson *et al.*, 1967)}.

Cell and mitochondrial matrix volumes were determined by subtracting either [^{14}C]carboxymethylinulin or [^{14}C]mannitol spaces, respectively, from the water space determined by ^3H_2O as described in Espié *et al.* (1995). Mitochondrial and cytosolic membrane electrical potential difference measurements in intact cells were performed using the equilibrium distribution of [^3H]$TPMP^+$ (triphenylmethylphosphonium ion) and $^{36}Cl^-$, respectively (Espié *et al.*, 1995).

Liver mitochondria were prepared according to Klingenberg and Slenczka (1959) in the following medium: 250 mM sucrose, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.2). Mitochondrial protein was estimated by the biuret method using bovine serum albumin as standard (Gornall *et al.*, 1949).

ATP/O ratios, with succinate as respiratory substrate, were determined from the ATP synthesis rate vs. respiratory rate with an ADP-regenerating system, based on hexokinase plus glucose. The rate of mitochondrial oxygen consumption was measured polarographically at 37°C using a Clark electrode. Respiration medium contained

Table I. Comparison between DNP Uncoupling and Effect of PUFA Deficiency in Liver Cells[a]

	J_{O_2} (nat O/min/mg dry mass)	Volume (μl/mg dry mass)		ATP (nmol/mg dry mass)		ADP (nmol/mg dry mass)		P_i (mM)		ATP/ADP-P_i (mM^{-1})		$\Delta\Psi$ Mito (mV)
		Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito	
Control	30.3 ± 1.1	0.98 ± 0.06	0.31 ± 0.02	6.8 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	4.6 ± 0.7	15.6 ± 0.3	1.51 ± 0.19	0.13 ± 0.01	-176 ± 4
PUFA deficiency	40.1 ± 1.5^{b}	0.69 ± 0.09^{b}	0.63 ± 0.03^{b}	6.4 ± 0.1^{b}	1.8 ± 0.1^{b}	1.5 ± 0.1^{b}	0.9 ± 0.1^{b}	8.5 ± 0.3^{b}	7.4 ± 0.3^{b}	0.58 ± 0.04^{b}	0.27 ± 0.01^{b}	-147 ± 8^{b}
Control + DNP	40.8 ± 1.5^{b}	0.95 ± 0.08^{c}	0.33 ± 0.01^{c}	$2.6 \pm 0.1^{b,c}$	$1.2 \pm 0.1^{b,c}$	$1.9 \pm 0.2^{b,c}$	0.7 ± 0.1^{c}	5.1 ± 0.9^{c}	14.9 ± 0.6^{c}	$0.35 \pm 0.04^{b,c}$	$0.11 \pm 0.01^{b,c}$	-151 ± 8^{b}

[a] Hepatocytes from control and polyunsaturated fatty acids (PUFA)-deficient rats were incubated in a Krebs bicarbonate medium containing 20 mM dihydroxyacetone and 4 mM octanoate with or without 50 μM 2,4-dinitrophenol (DNP). After a 25-min incubation, myxothiazol-sensitive oxygen uptake (J_{O_2}) was determined. At the same time, mitochondrial (mito) and cytosolic (cyto) adenine nucleotide and inorganic phosphate contents were measured. In parallel experiments, cell and mitochondrial matrix volumes were determined by subtracting, respectively, [^{14}C]carboxymethylinulin or [^{14}C]mannitol spaces from the water space determined by $^{3}H_2O$. $\Delta\Psi$ was determined from the [^{3}H]TPMP^{+} distribution. Results are mean ± S.E.M.; $n = 8$ from four different rats; $p < 0.05$.

[b] Versus control.

[c] Versus PUFA deficiency.

125 mM KCl, 1 mM EGTA, 5 mM Tris-P_i, 20 mM Tris-HCl (pH 7.2), supplemented with 5 mM succinate-Tris/0.5 mM malate-Tris plus 1.25 μM rotenone. ATP production was monitored from glucose 6-phosphate formation (Bergmeyer, 1974) in presence of 20 mM glucose, 1 mM $MgCl_2$, and 125 μM ATP. Oxygen consumption and ATP synthesis were modulated by addition of hexokinase (0.45 U/ml).

Determination of control coefficients of phosphate and adenine nucleotide carriers was assessed on rat liver mitochondria in a medium containing 125 mM KCl, 1 mM EGTA, 5 mM Tris-P_i and 1 mM ADP, with 5 mM succinate as respiratory substrate and 20 mM Tris-HCl (pH 7.2, 37°C). The use of control analysis is an adequate tool to identify the kinetic control exerted by one enzymic step on the flux through a metabolic pathway at a steady state (Heinrich and Rapoport, 1974; Kacser and Burns, 1973). The control coefficient of a given step involved in oxidative phosphorylation was based on the effects of a specific inhibitor of this step; the flux control coefficient was defined as:

$$C^J_{I_{\max}} = \left(\frac{\delta J/J}{\Delta I/I_{\max}}\right)$$

where J refers to the steady state flux and $I_{\max}$ is the amount of inhibitor required for total inhibition of the enzyme (Groen *et al.*, 1982b). We used the model of Gellerich and co-workers (1990) to determine the control coefficients. This method is based on nonlinear regression to fit the experimental data to a model, assuming noncompetitive and quasi-irreversible inhibition.

ATPase activity was measured at 37°C in a medium containing 125 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM ATP, and 20 mM Tris-HCl (pH 7.5). Before measurement, mitochondria were incubated for 2 min in 0.35% (w/v) Triton-X100 with or without oligomycin 1.25 μg/mg protein. Oligomycin-sensitive ATP hydrolysis was assessed by determination of P_i production (Summer, 1944).

Western blots (Towbin *et al.*, 1979) were performed as previously described (Boulay *et al.*, 1986), except that phosphate buffer saline was supplemented with 0.05% (w/v) Tween 20. The nitrocellulose sheets were treated with a rabbit antiserum against the synthetic peptide C-V-L-V-Y-D-E-I-K-K-F-V, corresponding to the C-terminal sequence of the beef heart mitochondrial adenine nucleotide carrier (ANC) (Marty *et al.*, 1992) and then incubated with horseradish peroxidase-conjugated protein A. The immunoreactive ANC protein was detected using the ECL detection reagents. For quantification, the autoradiography films were scanned and band intensities were determined by software analysis with NIH Image (National Institute of Health, Bethesda, Maryland).

ATP, ADP, P_i, 3-hydroxybutyrate dehydrogenase, and hexokinase were purchased from Roche (Meylan, France). Succinic acid, rotenone, EGTA, oligomycin, myxothiazol, DNP, and Triton-X100 were from Sigma (L'Isle d'Abeau, France). Tris, HCl, malic acid, dihydroxyacetone, glucose, and $MgCl_2$ were from Merck (Nogent, France), octanoate was from Jansen-chimica (Pantin, France), while ECL detection reagents and labeled compounds were from Amersham (Les Ulis, France).

Results are expressed as mean ± S.E.M. Statistical significant differences were assessed by ANOVA followed by Fisher's protected least-significant differences (PLSD) post hoc test or by unpaired Student t-test (Stat View, Abacus concepts, Inc., Berkeley, California, 1992).

RESULTS

Effects of PUFA Deficiency and of DNP on Oxidative Phosphorylation in Hepatocytes

In intact hepatocytes, PUFA deficiency leads to a significant increase in respiratory rate associated with a decrease in cytosolic ATP/ADP ratio, suggesting that an uncouplinglike effect has occurred (Piquet *et al.*, 1996). To mimic this condition we treated control hepatocytes with a concentration of DNP, causing a respiratory increase that closely matched that observed in hepatocytes prepared from PUFA-deficient animals (Table I). As expected, both DNP treatment and PUFA deficiency significantly lowered mitochondrial $\Delta\Psi$ *in situ*. Furthermore, *in situ* determination of mitochondrial volume confirmed the large increase seen in isolated PUFA-deficient mitochondria already reported (Fontaine *et al.*, 1996) as well as in the intact liver (Levin *et al.*, 1957; Smith and De Luca, 1964), which was not accompanied by changes of the total cellular volume.

A remarkable finding reported in Table I is the large decrease in matricial phosphate concentration of PUFA-deficient cells relative to both untreated or uncoupled control cells. Since the mitochondrial ATP/ADP ratio was unaffected (2.1 ± 0.1 vs. 2.0 ± 0.1, respectively, for controls and PUFA-deficient group, $n = 8$, NS), this change translates into a significant increase of the mitochondrial ATP/ADP-P_i ratio (Table I). This finding is in striking contrast with the dramatic decrease of the cytosolic ATP/ADP ratio (6.2 ± 0.5 vs. 4.2 ± 0.1, respectively, for controls and PUFA-deficient group, $n = 8$, $p < 0.05$), which matches the increased cytosolic phosphate concentration of the DNP-treated hepatocytes, resulting in

a significant decrease of the cytosolic ATP/ADP-P_i ratio. Hence, the effects of PUFA deficiency on cellular phosphate potentials are very different from those caused by DNP and, therefore, cannot be accounted for by a simple protonophoric uncoupling. This is in good agreement with the previous results showing that even in isolated mitochondria PUFA deficiency was indeed responsible for an effect, which cannot be explained by a pure protonophoric effect. Since these experiments were performed at 25°C, we repeated them at 37°C and found similar results (data not shown, but see Fontaine *et al.*, 1996). Taken together, these results rather suggest that PUFA deficiency may exert an effect on the adenine nucleotide and/or phosphate carriers.

Effects of PUFA Deficiency on Phosphate and Adenine Nucleotide Carriers, and on the Overall Phosphorylation Pathway in Isolated Mitochondria

The matricial P_i accumulation depends on the pH gradient (Azzone *et al.*, 1976; Coty and Pedersen, 1974; Ligeti *et al.*, 1985; Reynafarje and Lehninger, 1978). A decrease in phosphate gradient across the mitochondrial membrane in PUFA-deficient cells could be due to a thermodynamic change (*i.e.*, a lower pH gradient across the membrane) or to a kinetic effect of PUFA deficiency on the P_i carrier, or both. We, therefore, determined both the pH gradient across the inner membrane and the control coefficient exerted by the P_i carrier using mersalyl as the inhibitor (Coty and Pedersen, 1974). We found that state-3 ΔpH was 0.65 ± 0.10 and 0.35 ± 0.10 ($n = 6$; $p < 0.01$) in mitochondria from control and PUFA-deficient rats, respectively, a finding that can account for the decreased phosphate accumulation in mitochondria from the latter. The experiments of Fig. 1A illustrate the results of a typical titration with mersalyl of state-3 oxygen consumption in mitochondria isolated from control and PUFA-deficient rats. The experiments document a significant decrease in P_i carrier flux control coefficient in the PUFA-deficient group (0.22 ± 0.03 vs. 0.53 ± 0.03 for the control group, $n = 3$, $p < 0.05$). Note that the mersalyl concentration needed to obtain a complete inhibition of respiration coupled to ATP synthesis was the same for both groups (10.67 ± 0.41 vs. 10.50 ± 0.35 nmol mersalyl/mg protein for control group; $n = 3$; NS) indicating that PUFA deficiency does not affect the content of the carrier.

The significant decrease of phosphate potential across the mitochondrial membrane in PUFA deficient rats could also be explained by a kinetic effect and/or by a thermodynamic change at the step of the ANC. Considering the electrogenicity of adenine nucleotide exchange through the ANC, the decrease in $\Delta\Psi$ shown in

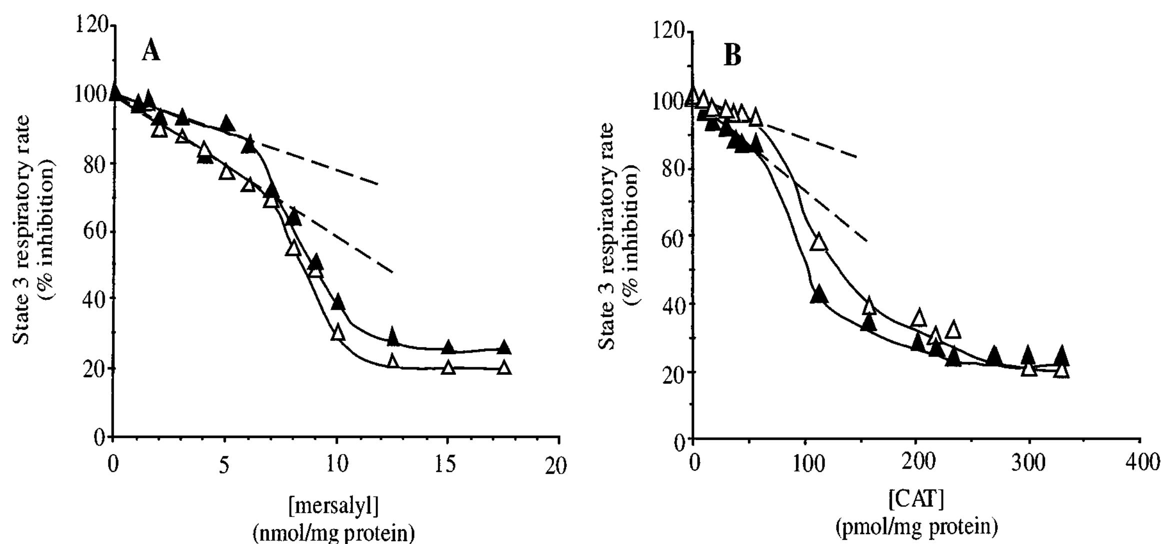

Fig. 1. Determination of flux control coefficient of phosphate carrier and adenine nucleotide carrier on state-3 oxygen consumption of liver mitochondria isolated from control and PUFA-deficient rats. Rat liver mitochondria (1 mg/ml) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 20 mM Tris-HCl (pH 7.2), supplemented with 5 mM Tris-succinate, 0.5 mM Tris-malate, 5 mM Tris-P_i, 1.25 μM rotenone, and 1 mM ADP. Respiratory rate, expressed as percentage of state-3 oxygen consumption, was inhibited by increasing addition of **(A)** mersalyl or **(B)** carboxyatractyloside (CAT). One typical experiment out of three is shown. Control (△), PUFA-deficient (▲).

Table I could provide an adequate explanation for the decreased gradient of ATP/ADP ratios (cytosol/matrix) across the mitochondrial membrane in PUFA deficient cells (2.2 ± 0.1 vs. 3.0 ± 0.2 for control groups, $n = 8$, $p < 0.05$). Yet, under these conditions the transport catalyzed by the ANC is out of equilibrium (Wanders *et al.*, 1981), and this step is probably controlled by kinetic rather than by thermodynamic parameters. Determination of ANC flux control coefficient was performed by using CAT as a quasi-irreversible inhibitor (Vignais *et al.*, 1973). Figure 1B shows the effect of PUFA deficiency on CAT inhibition of oxygen uptake under experimental conditions similar to those used for the P_i carrier. The control exerted by ANC was significantly increased in the PUFA-deficient group (0.52 ± 0.06 vs. 0.32 ± 0.03 for control group, $n = 3$, $p < 0.05$). It must be pointed out that also in this case the concentration of inhibitor required for maximal inhibition was identical in both groups (170 ± 19 vs 177 ± 15 pmol CAT/mg protein for control group, $n = 3$, NS). The apparent discrepancy between an increased flux control coefficient without a decrease of the number of binding sites led us to investigate the amount of protein by Western blotting. These experiments have been performed on isolated mitochondria with an anti-C-terminal peptide antiserum directed against ANC (Marty *et al.*, 1992). Figure 2 shows that the quantity of immunoreactive ANC is nearly unchanged in PUFA-deficient vs. control group ($84 \pm 14\%$ of control group content, $n = 16$, NS).

In the experiments of Table II, we studied mitochondria from either PUFA-deficient rats or from control rats with or without DNP addition in order to closely match the state-4 increased oxygen consumption rates as it is observed in the PUFA-deficient group. Phosphorylation was obtained by hexokinase addition permitting the simultaneous assessment of both oxygen consumption and ATP synthesis and calculation of ATP/O ratios. For a similar high respiratory rate, ATP/O ratio was only slightly affected in the PUFA-deficient group as compared to controls without DNP, while a profound decrease was observed in the presence of DNP. It must be noted that the ATPase activity was significantly increased in the PUFA-deficient group (719 ± 76 vs 413 ± 66 nmol P_i/min/mg proteins for the control group, $n = 5$; $p < 0.001$).

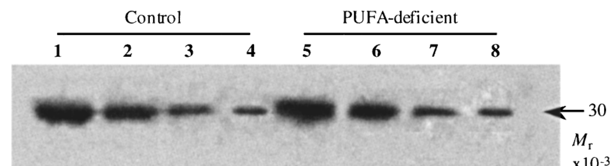

Fig. 2. Adenine nucleotide carrier (ANC) protein content in liver mitochondria isolated from control and PUFA-deficient rats. Samples of lysed mitochondria (20–2.5 μg protein) isolated from control (lanes 1–4) and PUFA deficient (lanes 5–8) rat livers were electrophoresed on a 15% polyacrylamide gel in presence of 0.4% SDS. After electroblotting, the nitrocellulose sheets were treated with antibodies directed to the C-terminal region of the bovine ANC. After incubation with horseradish peroxidase-conjugated protein A, the immunoreactive proteins were detected by a chemiluminescent assay. The antiserum specifically labeled a protein of molecular weight of about 30,000, corresponding to the ANC protein (Marty *et al.*, 1992). Lanes 1 and 5, 20 μg protein; lanes 2 and 6, 10 μg protein; lanes 3 and 7, 5 μg protein; lanes 4 and 8, 2.5 μg protein.

DISCUSSION

It has been established that PUFA deficiency was responsible for a decreased efficiency of respiratory chain in isolated mitochondria (Fontaine *et al.*, 1996). Moreover, it seems that the mechanism responsible for this is not limited to a pure protonophoric effect, but could involve a slippage of the redox proton pumps (Fontaine *et al.*, 1996). This conclusion was mainly based on the

Table II. Comparison between DNP Uncoupling and Effect of PUFA Deficiency in Liver Mitochondria[a]

	Without hexokinase	With hexokinase		
	J_{O_2} (natom O/min/mg protein)	J_{O_2} (natom O/min/mg protein)	J_{ATP} (nmol/min/mg protein)	ATP/O (natom/nmol)
Control	32 ± 1	133 ± 14	153 ± 21	1.14 ± 0.06
PUFA deficiency	51 ± 3^b	144 ± 8	136 ± 11^b	0.95 ± 0.05^b
Control + DNP	49 ± 1^b	135 ± 6	$61 \pm 17^{b,c}$	$0.44 \pm 0.12^{b,c}$

[a] Rat liver mitochondria (4 mg/ml) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 20 mM Tris-HCl (pH 7.2; 37°C), 5 mM Tris-P_i, supplemented with 5 mM Tris-succinate—0.5 mM Tris-malate plus 1.25 μM rotenone, 20 mM glucose, 125 μM ATP, and 1 mM $MgCl_2$. Oxygen consumption and ATP synthesis were modulated by addition of hexokinase (0.45 U/ml), with or without 3.75 μM 2,4-dinitrophenol (DNP). Results are mean ± S.E.M. ($n = 3$). $p < 0.05$.

[b] Versus control.

[c] Versus PUFA deficiency.

shape of the relationship between respiratory rate and pmf in nonphosphorylating mitochondria at 25°C. Similar results have been shown at 37°C (data not shown), thus confirming the existence of an energy wastage at the level of the respiratory chain. On the other hand, in intact liver cells, we found an increased oxygen consumption rate associated with a decreased in cytosolic ATP/ADP ratio (Piquet *et al.*, 1996). Although this could be explained by a "classical" protonophoric effect, it does not rule out other explanations.

The main finding of the present work is that in chronic PUFA deficiency, the increased mitochondrial energy dissipation caused by decreased respiratory chain coupling efficiency previously reported (Fontaine *et al.*, 1996) is offset by a series of *in vivo* compensatory changes in phosphorylation efficiency. These changes can be traced to a decreased P_i accumulation, an increased activity of ATP synthase, and an increased flux control coefficient by the ANC, which, in turn, allow maintenance of the ATP/ADP-P_i ratio. Indeed, the ATP/ADP-P_i ratio measured in hepatocytes from PUFA-deficient animals is much larger than that maintained by control hepatocytes treated with concentrations of DNP, matching the changes of respiration and $\Delta\Psi$. Despite identical rate of respiration and pmf, hepatocytes from PUFA-deficient animals are thus different from hepatocytes treated with uncoupler, a condition where the proton leak of the inner mitochondrial membrane has been enhanced.

Changes in mitochondrial volume appears to be a rather constant finding in the case of PUFA deficiency, since it has been reported in liver, isolated hepatocytes, and isolated liver mitochondria (Fontaine *et al.*, 1996; Levin *et al.*, 1957; Smith and De Luca, 1964). It has been shown in isolated hepatocytes, that mitochondrial volume changes were related to a decrease in mitochondrial $\Delta\Psi$ without affecting respiratory rate nor the ATP/ADP ratio in both matrix and cytosol (Espié *et al.*, 1995). Hence, the effect on mitochondrial volume of PUFA deficiency cannot explain the changes *per se* in oxidative phosphorylation.

Effect of PUFA Deficiency on Adenine Nucleotide Phosphorylation

P_i and adenine nucleotide transports, as well as ATPase activity, are affected by the PUFA deficiency and this could be due to either a kinetic or to a thermodynamic effect, or both. It has been shown that changes in membrane phospholipid composition affect the P_i carrier (Paradies and Ruggiero, 1991), the ANC (Streicher-Scott *et al.*, 1994), as well as the ATP synthase (Dabbeni-Sala *et al.*, 1981). P_i accumulation into the matrix is driven by the pH gradient across the inner membrane (Azzone *et al.*, 1976; Coty and Pedersen, 1974; Ligeti *et al.*, 1985; Reynafarje and Lehninger, 1978) and the significant fall in this gradient in PUFA deficiency explains the decreased matricial P_i accumulation. At the same time, we found a decrease in the flux control coefficient of the P_i carrier in PUFA deficiency, which is probably explained by direct activation of the carrier by the changes in lipid composition (Paradies and Ruggiero, 1991).

Since, on one hand, adenine nucleotide exchange is electrogenic, the decreased $\Delta\Psi$ may explain the decreased gradient of ATP/ADP ratios across the inner membrane in PUFA deficiency, which is consistent with the significant increase in ANC flux control coefficient (Fig. 1B). On the other hand, ANC is far from equilibrium (Wanders *et al.*, 1981). Therefore, it is important to stress that we failed to detect a significant change in ANC content in PUFA-deficient animals, as judged by both Western blotting with a specific antibody, or by titrations with carboxyatractylate, a specific ANC inhibitor that binds with a one-to-one stoichiometry (Vignais *et al.*, 1973). These findings rule out the possibility that the observed ANC kinetic changes of PUFA deficiency are due to changes of protein content. Rather they point to modulation by the phospholipid environment (Streicher-Scott *et al.*, 1994), which would be similar to that described for the P_i carrier.

The third step that was significantly affected in PUFA deficiency was the mitochondrial ATP synthase (+74%). Indeed, it has been reported that phospholipids of the inner mitochondrial membrane are able to stimulate the ATP synthase complex (Dabbeni-Sala *et al.*, 1974, 1981; Montecucco *et al.*, 1980; Pitotti *et al.*, 1972, 1980). In a reconstituted system, it was shown that negatively charged phospholipids are able to increase ATPase activity and that the reactivation of ATP synthase complex by phosphatidylcholine was dependent on both length and fluidity of acyl chains (Sandermann, 1978). Moreover, activation of ATPase by phospholipids seems also to depend on the conformation of adenine nucleotide translocator (Dabbeni-Sala *et al.*, 1981).

As a consequence of these multiple effects on the different steps of adenine nucleotide phosphorylation, it appears that in PUFA deficiency a high mitochondrial ATP/ADP-P_i ratio is maintained despite a lower Δp (Table I).

Effects of PUFA Deficiency on the Yield of Oxidative Phosphorylation

In the light of these adaptive processes at the level of the phosphorylation pathway, it is not too surprising that the net effects on the entire pathway (*i.e.*, the overall process of oxidative phosphorylation) may depend on

the experimental (or physiological) conditions. Indeed, our previous results show that in mitochondria isolated from PUFA-deficient rats, the ATP/O is unaffected at saturating ADP concentrations, while it is significantly decreased at constant subsaturating ADP concentrations (hexokinase regenerating system), a condition that mimics more closely the state of mitochondria *in situ* (Fontaine *et al.*, 1996). Hence, by using a similar experimental tool, it was of importance to compare the effect of PUFA deficiency with those obtained by a DNP uncoupling, precisely matching the state-4 respiratory rate increase resulting from PUFA deficiency. From Table II, it is obvious that the efficacy of oxidative phosphorylation is profoundly depressed with a protonophoric uncoupling (DNP), as compared to the effect of PUFA deficiency.

In summary, we propose that in chronic PUFA deficiency, a series of complex events take place. The initial event would be an increased respiratory rate not coupled to ATP synthesis and leading to a decrease of Δp. This would be followed by adaptive changes in the subsequent steps of phosphorylation, resulting in partial compensation of the coupling defect. This physiological adaptation permits the adverse effects of PUFA deficiency on respiratory chain efficiency to be counteracted. Hence, when the oxidative phosphorylation machinery is considered as a whole, the adaptive changes to PUFA deficiency permit the reaching of a different steady state, which is compatible with a normal life.

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