

NON-PHOSPHORYLATING RESPIRATION OF MITOCHONDRIA  
FROM BROWN ADIPOSE TISSUE OF RATS

by

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ABSTRACT

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Mitochondria from brown adipose tissue of cold-acclimated rats (6°C) oxidize  $\alpha$ -ketoglutarate at a rate twice that of controls (26°C). In both groups, however, the P/O ratio with  $\alpha$ -ketoglutarate has never exceeded unity, and is essentially zero with either succinate or  $\alpha$ -glycerophosphate. ATPase activity of these mitochondria is very low and is not stimulated by 2,4-dinitrophenol (DNP). Additionally, both respiration and phosphorylation are unaffected by ADP, DNP, bovine serum albumen or glutathione. Endogenous respiration of tissue slices is not stimulated by DNP. It is suggested that brown fat mitochondria are not capable of oxidative phosphorylation, but do phosphorylate at the substrate level. As these findings provide an unusual example of electron transport via an energetically non-conservative pathway, their significance to thermogenesis by brown adipose tissue is particularly emphasized.

In clean, tightly-coupled heart or liver mitochondrial systems, the P/O ratio in vitro is between 3.0 and 4.0 when  $\alpha$ -ketoglutarate is used as substrate. When the P/O ratio is reduced either by the presence of extra-mitochondrial material (1) or by the addition of an uncoupling agent such as 2,4-dinitrophenol (DNP) (2) the proportion of energy utilized in the formation of ATP is decreased relative to that directly dissipated as heat. Thus in brown adipose tissue, for which the primary known function is thermogenesis (3 - 8), an ideal metabolic state would appear to exist if the mitochondria maintained a high rate of respiration and a relatively low rate of phosphorylation. As it had been observed for some time in this laboratory that mitochondria from brown adipose tissue of rats phosphorylated only very poorly, it seemed of particular importance to investigate this in more detail.

Mitochondria from brown adipose tissue of adult male Long-Evans rats acclimated either at 26 or 6°C were isolated by a modification of the method of Schneider (9). The tissue was homogenized in 10 volumes of 0.25 M sucrose, and centrifuged at 14,000 x g for 10 minutes. After the supernatant was carefully aspirated from beneath the overlying lipid layer, the latter was removed and the walls of the tube swabbed out to eliminate as much lipid as possible. The pellet was resuspended in the original volume of 0.25 M sucrose and treated according to Schneider (9) for the isolation of the mitochondrial fraction ( $M_w$ ).

Oxygen consumption was measured at 37°C by the direct method of Warburg with air as the gas phase. The incubation medium for mitochondria contained, in  $\mu$ moles:  $\alpha$ -ketoglutarate, 10; KF, 10;  $PO_4$  as  $KH_2PO_4$  buffer (pH 7.4), 15; ATP, 2;  $MgCl_2$ , 5; tris buffer (pH 7.4), 25; glucose, 25 and 25 KM units hexokinase (Sigma Type IV). In experiments where ADP was added, the glucose-hexokinase trap was omitted. Total reaction mixture volume was 1.0 ml, including 0.4 - 0.5 ml mitochondria (ca. 0.3 mg N). The center well contained 0.05 ml 10% KOH and folded filter paper wick. Following a 12 minute temperature equilibration, trap or ADP ( $4 \times 10^{-3}M$  final concentration) was tipped into the vessel and readings were taken at 5 minute intervals for 15 minutes. Reactions were stopped by the addition of 0.5 ml 25% trichloroacetic acid and phosphate assays performed on the contents of each Warburg flask using the method of Gomori (10). Endogenous respiration of tissue slice was measured by the same method in a medium essentially the same as that used for  $M_w$ , but omitting substrate and glucose-hexokinase trap and substituting KCl for KF. DNP ( $4 \times 10^{-5}M$  final concentration) was tipped in at the time of the first reading. Readings were taken at 5 minute intervals for 20 minutes. Nitrogen was assayed by a standard micro-Kjeldahl method.

ATPase activities were assayed in a medium containing, in  $\mu$ moles: ATP, 15;  $MgCl_2$ , 5; KF, 10;  $PO_4$  as  $KH_2PO_4$  buffer (pH 7.4), 15; glucose, 25 and tris buffer (pH 7.4), 25. The final volume was 1.0 ml, including 0.4 ml mitochondria. Reactions were carried out at 37°C for 12 minutes.

The results summarized in Table 1 show that brown fat mitochondria from cold-acclimated rats oxidize  $\alpha$ -ketoglutarate at a rate twice that of

the control rats. This increase in  $qO_2$ , coupled with the increased mass of tissue in the cold (5, 11), contributes significantly to the total heat production (5) assignable to brown fat in the cold-acclimated animal.

Also notable are the low P/O ratios observed in the mitochondria from both the control and cold-acclimated animals (Table 1). These low ratios do not appear to result from ATPase activities, which in fact are very low and moreover are not stimulated in vitro by addition of DNP (Table 2). However, it has been noted that oligomycin (2  $\mu$ g/mg protein) effected approximately 90% inhibition of the ATPase activity.

Thus far, all attempts to restore phosphorylation in brown fat mitochondria have been unsuccessful. Neither bovine serum albumin (BSA) (6 or 15 mg/flask) nor glutathione ( $10^{-3}M$ ), when added to the Warburg flasks, enhances the P/O ratio. Moreover, the presence of BSA in the homogenization medium (6 mg/ml) has not affected the P/O ratios of the isolated mitochondria, suggesting that these low ratios do not result from an uncoupling effect of fatty acids (12) released upon homogenization (Table 3).

Experiments with other substrates indicate that mitochondria isolated from brown adipose tissue do not yield theoretical, or even near-theoretical, P/O ratios. Initial rates of respiration and resultant P/O ratios determined with a polarized platinum electrode have yielded results identical to those reported here (13). Since the P/O ratios in systems employing  $\alpha$ -glycerophosphate and succinate are essentially zero (respectively,  $0.086 \pm 0.028$  and  $0.111 \pm 0.023$ ), the P/O ratio of near unity observed with  $\alpha$ -ketoglutarate may represent phosphorylation at the substrate level. This interpretation is further substantiated by the finding that addition of DNP (2 or 4  $\times 10^{-5}M$ ) does not significantly affect the phosphorylation or the respiration (Table 3). It is known (14) that the substrate level phosphorylation with  $\alpha$ -ketoglutarate is insensitive to DNP. Moreover, with  $\alpha$ -ketoglutarate in the absence of either glucose-hexokinase trap or ADP, P/O ratios were zero; addition of ADP, however, did result in phosphorylation, but the values only approached those obtained in the presence of the trap.

Although the inhibitory effect of oligomycin on the mitochondrial ATPase activity may be interpreted as evidence for the presence of a phosphorylating mechanism usually associated with the electron transport system (15), the studies on tissue slices fail to show any stimulation of respiration by DNP either in control or cold acclimated animals. The ratios of respiration (+DNP/-DNP) were 0.86 and 1.09 in the control and cold acclimated tissue slices, respectively. These results strongly suggest that the lack of oxidative phosphorylation exhibited by the mitochondrial preparations may not be artifact of isolation, but rather may reflect an absence of coupled oxidative phosphorylation in vivo.

If indeed the usual oxidative phosphorylation mechanism does not operate in this highly respiring, heat producing tissue, requirements for ATP might still be met by substrate phosphorylations such as that suggested here with respect to  $\alpha$ -ketoglutarate, and those presumably occurring via

the glycolytic pathway. Thus, if in vivo the P/O ratio and  $qO_2$  of  $\alpha$ -ketoglutarate oxidation were of the same order as seen in vitro, this would yield, in the control rat, 17.1  $\mu$ moles ATP/mg N hr and in the cold-acclimated rat, 29.1  $\mu$ moles ATP/mg N hr. In 350 - 400 gm rats, the total mitochondrial nitrogen of the brown fat is 1.95 mg for control rats and 8.67 mg for cold-acclimated rats (16). The total amount of ATP formed by the brown fat would then be 33.5  $\mu$ moles/hr in the control and 251.9  $\mu$ moles/hr in the cold-acclimated rat. Hence, solely on the basis of substrate level phosphorylation with  $\alpha$ -ketoglutarate, ATP formation in the brown fat of cold-acclimated rats could be increased 750% over that in controls.

Significant in the present findings are a) the apparent absence of a system coupling respiration with phosphorylation and b) the resulting lack of respiratory control by ADP. The former eliminates the necessity of synthesizing a high energy intermediate or compound, which if this were a coupled system, would occur in large amounts, while the absence of ADP respiratory control would insure a high rate of substrate oxidation. The resulting effect would therefore maximize the rate of production and availability of heat evolved by the respiring system. This, together with the increased  $qO_2$  of the mitochondria of brown fat from cold-acclimated rats, is believed to bear primarily upon the augmentation of heat production in this tissue. Also, these findings are in concert with the known physiological role of brown adipose tissue as a thermogenetic effector organ both in cooled or cold-exposed mammals and in hibernators during cold-induced arousal (3 - 8).

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Table 1. Oxygen consumption and P/O ratios of brown fat mitochondria  
(Substrate =  $\alpha$ -ketoglutarate).

	n	$\dot{q}O_2$ ( $\mu l$ $O_2$ /mg N hr)	P/O
Control	9	$303.38 \pm 30.13^*$	$0.633 \pm 0.066$
Cold	15	$606.20 \pm 38.28$	$0.537 \pm 0.020$
P <sub>control vs cold</sub>		$< 0.001$	n.s.

\*mean  $\pm$  S.E.

Table 2. ATPase activities of brown fat mitochondria, with and without addition of DNP, expressed as  $\mu$ moles ATP hydrolyzed/mg N hr.

	n	- DNP	+ DNP ( $4 \times 10^{-5}M$ )	P
Control	3	$33.52 \pm 11.62^*$	$43.07 \pm 8.99$	n.s.
Cold	3	$26.86 \pm 1.85$	$34.61 \pm 5.86$	n.s.

\* mean  $\pm$  S.E.



Table 3. Effects of DNP, ADP and BSA on oxygen consumption and P/O ratios of brown fat mitochondria from cold acclimated rats. (Substrate =  $\alpha$ -ketoglutarate).

Agent	n	$\dot{q}O_2$		P/O	
		no agent	+ agent	no agent	+ agent
DNP ( $2 \times 10^{-5}M$ )	3	535.66 $\pm$ 39.59*	502.27 $\pm$ 49.23	0.518 $\pm$ 0.039	0.594 $\pm$ 0.094
DNP ( $4 \times 10^{-5}M$ )	3	535.66 $\pm$ 39.59	522.30 $\pm$ 32.56	0.518 $\pm$ 0.039	0.464 $\pm$ 0.054
ADP ( $4 \times 10^{-3}M$ )	3	476.30 $\pm$ 15.45	460.42 $\pm$ 23.21	0	0.369 $\pm$ 0.039
BSA (6 mg/ml homogeniz- ation medium)	3	733.30 $\pm$ 83.72	751.51 $\pm$ 125.70	0.535 $\pm$ 0.039	0.642 $\pm$ 0.029

\* mean  $\pm$  S.E.