Nonphosphorylating Respiration of Mitochondria from Brown Adipose Tissue of Rats

Abstract. *Mitochondria from brown adipose tissue of cold-acclimated rats (6°C) oxidize α-ketoglutarate at a rate twice that of controls (26°C). In both groups, however, the phosphorus : oxygen ratio with α-ketoglutarate never exceeded unity, and it is essentially zero with either succinate or α-glycerophosphate. Adenosine triphosphatase activity of these mitochondria is very low and it is not stimulated by 2,4-dinitrophenol. In addition, both respiration and phosphorylation are unaffected by adenosine diphosphate, 2,4-dinitrophenol, bovine serum albumin, or glutathione. Endogenous respiration of tissue slices is not stimulated by 2-4-dinitrophenol. It is suggested that brown fat mitochondria are not capable of oxidative phosphorylation, but do phosphorylate at the substrate level. Since these findings provide an unusual example of electron transport by means of an energetically nonconservative pathway, their significance to thermogenesis by brown adipose tissue is particularly emphasized.*

In clean, tightly coupled heart or liver mitochondrial systems, the phosphorus : oxygen ratio in vitro is between 3.0 and 4.0 when α-ketoglutarate is used as substrate. When the P : O ratio is reduced either by the presence of extramitochondrial 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 adenosine triphosphate (ATP) is decreased in relation to the energy dissipated as heat. Thus, in brown adipose tissue, for which the primary known function is thermogenesis (*3, 4*), an ideal metabolic state would appear to exist if the mitochondria maintained a high rate of respiration and a relatively low rate of phosphorylation. Since it had been observed earlier (*5*) that mitochondria from brown adipose tissue of rats phosphorylated only very poorly, it seemed of particular importance to investigate this finding in more detail.

Mitochondria from brown adipose tissue of adult, male Long-Evans rats acclimated at either 26° or 6°C were isolated by a modification of the method of Schneider (*6*). The tissue was homogenized in 10 volumes of 0.25*M* sucrose and centrifuged at 14,000*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 were 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 (*6*) for the isolation of the mitochondrial fraction (M_w). Rat liver mitochondria prepared by this method consistently yielded P : O ratios near 3 with α-ketoglutarate (3.37) and β-hydroxybutyrate (2.96).

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 micromoles, α-ketoglutarate, 10; KF, 10; phosphate as KH_2PO_4 buffer (*p*H 7.4), 15; ATP, 2; $MgCl_2$, 5; tris buffer (*p*H 7.4), 25; glucose, 25; and 25 K_m units hexokinase (Sigma Type IV). In experiments in which ADP was added, the glucose-hexokinase trap was omitted. The volume of the total reaction mixture was 1.0 ml, including 0.4 to 0.5 ml of mitochondria (about 0.3 mg of nitrogen). The center well contained 0.05 ml of 10-percent KOH and a folded filter paper wick. After a 12-minute period of 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 adding 0.5 ml of 25-percent trichloroacetic acid, and phosphate assays were performed on the contents of each Warburg flask using the method of Gomori (*7*). Endogenous respiration of tissue slice was measured by the same method in a medium that was essentially the same as that used for M_w but with omission of substrate and glucose-hexokinase trap and substitution of KCl for KF. 2-4-Dinitrophenol ($4 \times 10^{-5}M$ 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.

Adenosine triphosphatase activities were assayed in a medium that contained, in micromoles, ATP, 15; $MgCl_2$, 5; KF, 10; phosphate as KH_2PO_4 buffer (*p*H 7.4), 15; glucose, 25; and tris buffer (*p*H 7.4), 25. The final volume was 1.0 ml, including 0.4 ml of mitochondria. Reactions were carried out at 37°C and stopped after 12 minutes by adding 0.5 ml of 25-percent trichloroacetic acid. The method of Gomori (*7*) was used to determine the appearance of inorganic phosphate. Zero time controls were used to account for the phosphate buffer that was present in the incubation medium.

The results summarized in Table 1 show that brown fat mitochondria from cold-acclimated rats oxidize α-ketoglutarate at a rate twice that of the control rats. This increase in oxygen quotient, coupled with the increased mass of tissue in the cold-acclimated rats (*3, 8*), contributes significantly to the total heat production (*3*) that can be assigned to brown fat in the cold-acclimated animal.

Also notable are the low P : O ratios that are observed in the mitochondria from both the control and the cold-acclimated animals (Table 1). These low ratios do not appear to result from adenosine triphosphatase activities, which, in fact, are very low and are not stimulated in vitro by the addition of DNP (Table 2). However, it has been noted that oligomycin (2 μg/mg of

Table 1. Oxygen consumption and P : O ratios of brown fat mitochondria.

Number	Oxygen quotient (microliters of oxygen per milligram of nitrogen per hour)	P : O ratios
	Control rats	
9	303.4 ± 30.1*	0.633 ± 0.066
	Cold-acclimated rats	
15	606.2 ± 38.3	0.537 ± 0.020
	Significant levels (control versus cold-acclimated rats)	
	<0.001	Not significant

* Mean ± standard error.

Table 2. Adenosine triphosphatase activities of brown fat mitochondria, with and without addition of DNP, expressed as micromoles of ATP hydrolyzed per milligram of nitrogen per hour. There are no significant differences between adenosine triphosphatase activities with and without DNP.

Number	Without DNP	With DNP ($4 \times 10^{-5}M$)
	Control rats	
3	33.5 ± 11.6*	43.1 ± 8.9
	Cold-acclimated rats	
3	26.9 ± 1.8	34.6 ± 5.9

* Mean ± standard error.

Table 3. Effects of DNP, ADP and BSA on oxygen consumption and P : O ratios of brown fat mitochondria from cold-acclimated rats (substrate, α-ketoglutarate).

Rats (No.)	Oxygen quotient		P : O ratios	
	No agent	With agent	No agent	With agent
		DNP (2×10^{-5}M)		
3	535.7 ± 39.6*	502.3 ± 49.2	0.518 ± 0.039	0.594 ± 0.094
		DNP (4×10^{-5}M)		
3	535.7 ± 39.6	522.3 ± 32.6	0.518 ± 0.039	0.464 ± 0.054
		ADP (4×10^{-3}M)		
3	476.3 ± 15.4	460.4 ± 23.2	0	0.369 ± 0.039
		BSA (6 mg/ml homogenization medium)		
3	733.3 ± 83.7	751.5 ± 125.7	0.535 ± 0.039	0.642 ± 0.029

* Mean ± standard error.

protein) effected approximately a 90-percent inhibition of the adenosine triphosphatase activity.

All attempts to restore phosphorylation in brown fat mitochondria have been unsuccessful. Neither bovine serum albumin (BSA) (6 or 15 mg per flask) nor glutathione ($10^{-3}M$) enhances the P:O ratio when they are added to the Warburg flasks. 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 (*9*) 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 the resulting P:O ratios as determined with a polarized platinum electrode have yielded results identical to those reported here (*10*). Since the P:O ratios in systems employing α-glycerophosphate and succinate are essentially zero (0.086 ± 0.028 and 0.111 ± 0.023, respectively), the P:O ratio of near unity that has been observed with α-ketoglutarate may represent phosphorylation at the substrate level. This interpretation is further substantiated by the finding that the addition of DNP (2 or $4 \times 10^{-5}M$) does not significantly affect phosphorylation or respiration (Table 3). It is known (*11*) that the substrate level phosphorylation with α-ketoglutarate is insensitive to DNP. Moreover, with α-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 adenosine triphosphatase activity may be interpreted as evidence for the presence of a phosphorylating mechanism usually associated with the electron transport system (*12*), 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 an 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 α-ketoglutarate, and those presumably occurring by means of the glycolytic pathway. Thus, if in vivo the P:O ratio and the oxygen quotient of α-ketoglutarate oxidation were of the same order as seen in vitro, this would yield 17.1 μmole/hr of ATP per milligram of nitrogen in the control rat, and 29.1 μmole/hr of ATP per milligram of nitrogen in the cold-acclimated rat. In 350- to 400-g rats, the total mitochondrial nitrogen of the brown fat is 1.95 mg for control rats and 8.67 mg for cold-acclimated rats (*13*). The total amount of ATP formed by the brown fat would then be 33.5 μmole/hr in the control rats and 251.9 μmole/hr in the cold-acclimated rats. Hence, solely on the basis of substrate level phosphorylation with α-ketoglutarate, ATP formation in the brown fat of cold-acclimated rats could be increased 750 percent over that in controls.

Significant in the present findings are (i) the apparent absence of a system coupling respiration with phosphorylation and (ii) 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 oxygen quotient 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 agree 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 (*3–4*).

Note added in proof: Since submitting this report, we have learned from O. Lindberg that, in using brown adipose tissue from newborn rabbits, he obtained results similar to those reported here with respect to the apparent absence of classical electron-transport-coupled phosphorylation.

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