RESPIRATION-LINKED PHOSPHORYLATION IN MITOCHONDRIA OF GUINEA-PIG BROWN FAT

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Uncoupling of oxidative phosphorylation has been proposed as the cause of the great thermogenic capacity of brown adipose tissue (brown fat).

The low P:O ratios exhibited by brown fat mitochondria under usual experimental conditions have been interpreted as representing substrate-linked phosphorylation by some workers (Lindberg et al. 1967;Smith et al. 1966;Thomson et al. 1968). In contrast, others (Guillory and Racker, 1968;Hohorst and Stratmann, 1967;Prusiner et al. 1968a,b) have produced evidence which suggests that such mitochondria are able to carry out oxidative phosphorylation in an apparently normal fashion. It must be emphasized that these conflicting results derive from experiments with several species at different ages and under varying experimental conditions.

Serious consideration has recently been given to the possibility that brown fat mitochondria carry defective oxidative phosphorylation (Kornacker and Ball, 1968),and it has been suggested that ATP generation occurs at only one of the three usual sites (Aldridge and Street, 1968).

It was considered important therefore to investigate the

details of this presumed defective respiration-linked phosphorylation and its possible physiological significance.

METHODS: Interscapular brown fat tissue of new-born (less than 24 hours' old) and 3 weeks' old guinea-pigs was gently homogenized in a medium consisting of 0.33 M sucrose-1 mM HEPES buffer pH 7.4 (1:3 w/v dilution), with a loose-fitting teflon pestle in a polypropylene centrifuge tube. The homogenate was centrifuged at 4500 g.min.(Sorvall rotor SS-34) and the procedure was repeated once (3000 g.min.). The combined supernatants were centrifuged at 32000 g.min (Spinco rotor 40.3), and the resulting mitochondrial fraction was suspended in the homogenization medium. All manipulations were performed at 0-4°C.

Oxidative phosphorylation was determined by manometry or spectrophotometry using the procedures described in the legend to Table I. Incorporation of phosphate was measured essentially according to Lindberg and Ernster (1956). n-Hexanol was substituted for isobutanol-benzene, since this has been shown more effective in extracting the phosphomolybdate complex (Hagihara and Lardy, 1960). The non-extracted ^{32}P was estimated by counting in a Packard Tricarb scintillation counter. Protein was determined according to Lowry et al. (1951). Respiratory control ratios were determined by manometry or by using a Clark oxygen electrode system (Yellow Springs Instruments Biological Oxygen Monitor).

Mitochondria of guinea-pig brains were prepared as described above along with brown fat mitochondria from the same animals and carried as controls in the subsequent experiments.

RESULTS AND DISCUSSION: Mitochondria from new-born guinea pig brown fat exhibited low, but significant phosphorylation

TABLE I Oxidation rates and P:O ratios observed with mitochondria of brown fat and brain of new-born guinea-pigs

Reaction	Brown fat		Brain	
	Specific oxidation rate†	P:O	Specific oxidation rate	P:O
Pyruvate-malate→O_2	58.8 ± 11.7	0.47 ± 0.16 (8)	35.4 - 30.8	2.56 - 1.97
DL-β-hydroxybutyrate→O_2	18.0 ± 4.5	0.24 ± 0.05 (4)	13.5 - 12.5	1.60 - 2.02
DL-β-hydroxybutyrate→O_2††	15.2 - 15.8	0.19 - 0.18	10.5	2.34
DL-β-hydroxybutyryl-L-carnitine→O_2	82.0 - 82.3	0.20 - 0.20	14.5	1.60
Succinate→O_2	105.6 ± 15.5	0.08 ± 0.03 (6)	36.6 ± 6.1 (3)	1.53 ± 0.23
Succinate $\xrightarrow[\text{antimycin}]{\text{TMPD}}$ O_2	87.2 - 85.9	0.02 - 0.03	132.0	0.37
Succinate $\xrightarrow[\text{antimycin}]{\text{TMPD}}$ O_2†††	78.6 - 85.4	0.04 - 0.03	32.5	0.48
Ascorbate-TMPD→O_2†††	112.0 -125.0	0.04 - 0.04	53.2	0.72
Pyruvate-malate→$Fe(CN)_6^{3-}$	4.82- 4.81	0.37 - 0.37	3.5	1.00
Succinate→$Fe(CN)_6^{3-}$	5.48± 0.12	0.03 ± 0.006 (3)	2.77 - 2.86	0.36 - 0.26

† muatoms oxygen/mg protein/min. or oxidation of $Fe(CN)_6^{3-}$ calculated as equivalents to O (2e).
†† In the presence of 0.1 mM CoA and 1 mM DL-carnitine. ††† No cytochrome c added to the reaction medium. When more than two experiments are presented the results are given as mean ± s.d. The number in parentheses indicate the number of experiments.

under the prevailing conditions (Table I). The P:O ratio found with pyruvate-malate as substrate may represent both substrate- and respiratory chain-linked phosphorylation (sites I +II + III). The difference between this figure and that obtained with DL-β-hydroxybutyrate suggests that part at least is respiratory chain-linked. It is generally assumed that there is no substrate phosphorylation linked to the oxidation of DL-β-hydroxybutyrate (Borgström et al. 1955). Compared to the free acid, DL-β-hydroxybutyryl-L-carnitine showed considerably (6-fold) enhanced oxidation with brown fat mitochondria, but accompanied by a nearly identical P:O ratio; this contrasts with the results with brain mitochondria.

Manometric measurements were carried out at 30°C in a medium containing 10 mM potassium phosphate buffer,pH 7.4, with 333 mμc ^{32}P/μmole P_i; 5 mM $MgCl_2$; 60 mM HEPES (Calbiochem) buffer, pH 7.4; 1% (w/v) bovine serum albumin (Mann Research, fatty acid-poor); 220 mM sucrose; 30 μM cytochrome c; 2 mM ATP; 50 mM glucose; 11 units/ml of hexokinase (Sigma), in a total volume of 2.0 ml. Substrates were used at the following concentrations: 5 mM pyruvate and 5 mM malate in the presence of 0.25 mM NAD; 10 mM DL-β-hydroxybutyrate; 6 mM DL-β-hydroxybutyryl-L-carnitine; 10 mM succinate (+ 0.6 μM rotenone); 20 mM ascorbate (+ 0.6 μM rotenone). The TMPD concentration was 0.4 mM, antimycin being used as 1 μg/mg of mitochondrial protein. Mitochondrial protein was 4-8 mg/vessel and the respiration was measured for 15-20 minutes. The ascorbate/TMPD system for site III phosphorylation was essentially that of Sanadi and Jacobs (1967), and the succinate/antimycin/TMPD system for the same site was that of Lee et al. (1967) adapted to manometry. The ferricyanide method was modified after Schatz and Racker (1966). These reactions were carried out at 30°C in a total volume of 3.0 ml. The incubation medium and substrate concentrations were as above except that cytochrome c was omitted, the sucrose concentration was 190 mM, and that 1.5 mM KCN was added. After 5 minutes of preincubation, the reaction was started by the addition of $K_3Fe(CN)_6$ to a final concentration of 0.8 mM. Mitochondrial protein was 2 mg/cuvette and the reduction of ferricyanide was followed spectrophotometrically for 5 minutes. In calculating the P:2e ratio, correction was made for the non-phosphorylating antimycin-insensitive reduction of the ferricyanide (which amounted to approximately 20% of the reduction found in the absence of antimycin).

Further analysis of the phosphorylation sites of brown fat mitochondria revealed that uncoupling may apply to all three sites, while sites II and III were the more strongly affected. Thus all of the following reactions were accompanied by insignificant P:O ratios: Succinate→O_2(sites II + III); succinate →$Fe(CN)_6^{3-}$ (site II); ascorbate-TMPD→O_2 (site III); succinate→O_2 shunted by TMPD over the antimycin-inhibited site (site III). The results further suggest that the measurable phosphorylation linked to the terminal part of the respiratory chain is equally distributed between sites II and III. Endogenous respiration was not detected.

Several investigators (Aldridge and Street, 1968; Guillory and Racker, 1968) have stressed the benevolent effects of serum albumin on the phosphorylating capacity of brown fat mitochondria. Under our experimental conditions, the presence of 1% bovine serum albumin (Mann Research, fatty acid-poor) in the homogenization medium had no demonstrable effect on P:O ratios or respiration rates with a variety of substrates. EDTA (1-2 mM) in the homogenization medium likewise was without effect. Moreover, the presence of 1% serum albumin (extensively defatted according to Guillory and Racker (1968)) in the incubation media, although leading to improved respiratory rates, did not appear to affect strongly the P:O ratios associated with the pyruvate-malate→O_2 or succinate→O_2 reactions. However, detection of phosphorylation with ferricyanide proved absolutely dependent on the presence of serum albumin in the incubation medium, again accompanied by a 3-fold stimulation of the respiratory rate.

In contrast to the situation in new-born guinea-pig brown fat mitochondria, those of 3 weeks' old animals revealed near normal oxidative phosphorylation (Table II). This striking

TABLE II

Oxidation rates and P:O ratios observed with brown fat mitochondria of 3 weeks' old guinea-pigs

Reaction	Specific oxidation rate	P:O
Pyruvate-malate→O_2	54.5 ± 21.2 (3)	2.60 ± 0.61
Succinate→O_2	120.3 ± 19.9 (3)	0.93 ± 0.04
Succinate-$\xrightarrow[\text{antimycin}]{\text{TMPD}}$$O_2$	51.5 ± 1.0 (3)	0.05 ± 0.01
Ascorbate-TMPD→O_2	159.1 ± 3.3 (3)	0.05 ± 0.01

Experimental conditions as described in the legend to Table I.

difference may represent a phenomenon of physiological significance. In fact, under identical experimental conditions, since brown fat cells of 3 weeks' old animals contain a much higher proportion of fat, procedural or free fatty acid uncoupling would be expected to be more prominent in the mitochondria of older animals. Contrary to this expectation, the evidence strongly suggests the establishment of normal phosphorylation capacity linked to site II (as represented by the P:O ratio difference of the succinate→O_2 and succinate-$\xrightarrow[\text{antimycin}]{\text{TMPD}}$$O_2$ or succinate→O_2 and ascorbate-TMPD→O_2 reactions). Similarly, Table II reveals that the brown fat mitochondria of the older animals possess fully coupled phosphorylation at site I (the P:O ratio difference of the malate-pyruvate→O_2 and succinate→O_2 reactions). Site III phosphorylation in these mitochondria remains virtually uncoupled (succinate-$\xrightarrow[\text{antimycin}]{\text{TMPD}}$$O_2$ and ascorbate-TMPD→O_2 reactions).

Low, and nearly identical respiratory control ratios were

exhibited by mitochondria from both new-born and 3 weeks' old guinea-pigs (Table III). Pyruvate-malate oxidation showed clear control while the oxidation of succinate and ascorbate-TMPD proceeded with no or barely measurable ADP control.

The following pretreatments or additions to the homogenization or incubation media failed to elicit additional respiratory control: EDTA, ATP, GTP, and additions of serum albumin to the homogenization medium (Hohorst and Rafael, 1968).

TABLE III

Respiratory control ratios† of brown fat mitochondria

Substrates	Manometry††		Polarography †††	
	new-born	3 wks old	new-born	3 wks old
Pyruvate-malate	2.6	2.5	1.2	1.3
Succinate	1.1	1.1	1.0	1.05
Ascorbate-TMPD	-	-	1.0	1.0

† Respiratory control ratio = $\frac{\text{respiratory rate with ADP}}{\text{respiratory rate without ADP}}$

†† The protein concentration was 2 mg/ml; 2 mM ADP; 30°C.

††† Protein concentrations were 0.5 mg/ml and 0.2 mg/ml for new-born and 3 weeks' old animals, respectively; 0.1 mM ADP; 25°C.

The incubation medium was that described in the legend to Table I, except that the glucose-hexokinase system was omitted.

Similarly, addition of 0.2 mM Ca^{2+} to mitochondria free of extramitochondrial Ca^{2+} failed to stimulate respiration. Mitochondria of guinea-pig brown fat therefore do not exhibit normal characteristics of states 3 and 4 oxidations (Chance, 1959). The observation that the establishment of near normal respiration-linked phosphorylation in animals aged 3 weeks was

not accompanied by improved respiratory control again favours the presence in brown adipose tissue of a specialized type of mitochondrion.

That these findings were not attributable to damaged mitochondria is supported by the fact that the succinate-ferricyanide system was effectively blocked by antimycin (80% under our experimental conditions), this being taken to represent a phenomenon exhibited exclusively by mitochondria in the intact condition (Lee et al. 1967).

In conclusion, our results suggest that brown fat mitochondria of new-born guinea-pigs are capable of respiration-linked phosphorylation, though of small magnitude. Part of this is substrate-linked. The respiratory chain-linked phosphorylation is coupled to site I, and insignificantly to sites II and III. The striking increase in phosphorylation capacity of 3 weeks' old guinea-pig brown fat mitochondria justify the assumption that the uncoupling found in new-born animals may have significance related to the physiological function of this tissue. Moreover, the implications of coincident existence in brown fat tissue of thermogenesis and partially uncoupled phosphorylation have not escaped our notice.

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