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STUDIES ON THE ENERGY STATE OF ISOLATED BROWN ADIPOSE TISSUE MITOCHONDRIA

EFFECT OF ADENINE NUCLEOTIDES AND OLIGOMYCIN ON THE GENERATION AND DISSIPATION OF THE "ENERGY POTENTIAL"

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SUMMARY

Energization of isolated brown adipose tissue mitochondria of cold-stressed guinea pigs has been studied by measuring rates and steady-state reduction of the cytochrome *b* complex. Our previous conclusion (Pedersen, J. I. and Flatmark, T. (1972) *Biochim. Biophys. Acta* 275, 135–147) that brown adipose tissue and liver mitochondria of these animals are fundamentally different from an energetic point of view, has been confirmed.

ADP induced an energization of brown adipose tissue mitochondria very similar to that previously observed with ATP (ref. cited), but the maximal "energy potential" obtained by ADP is lower. Furthermore, this potential of brown adipose tissue mitochondria is much more sensitive to changes in the extramitochondrial phosphate potential than is that of liver mitochondria. Energization by ADP is largely mediated by ATP formed by the adenylate kinase reaction.

The oligomycin-induced oxidation of the cytochrome *b* complex of maximally energized mitochondria appears to be a suitable measure of the rate of energy dissipation. By using this parameter, it has been found that the rate as well as the extent of endogenous dissipation of energy is approx. 15 times higher in brown adipose tissue mitochondria than in liver mitochondria at pH 6.8. The pH dependence of this reaction is a further indication of the importance of the transmembrane pH gradient in the control of coupling of electron transport to phosphorylation in brown adipose tissue mitochondria.

INTRODUCTION

Studies of the respiratory activities[1] as well as of the energy dependent calcium uptake[2] in isolated brown adipose tissue mitochondria from cold exposed guinea pigs have revealed that these mitochondria are loosely coupled. The loosely coupled state

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

has been found to be associated with a low intramitochondrial concentration of ATP and P_i and a high concentration of AMP[1]. Together with a striking effect of high-energy nucleotides on the respiratory control[3–5] this observation has lead to the suggestion that the mitochondrial nucleotide pool plays an important role in the control of coupling of electron transport to phosphorylation in these mitochondria. The mechanism of this control is, however, still unclear.

By using the cytochrome *b* complex as a probe of the "energy potential"* of the mitochondrial inner membrane[7,8] we have previously found that brown adipose tissue mitochondria differ markedly from liver mitochondria[7]. Thus, freshly isolated brown adipose tissue mitochondria are deenergized and the energy dependent reductions of the cytochromes *b*** require considerably higher ATP concentrations than in liver mitochondria. Furthermore, these reductions are strongly pH dependent.

In the present work further studies on the effect of adenine nucleotides and oligomycin on the energy state of isolated brown adipose tissue mitochondria have been undertaken. The results obtained indicate that the thermogenic function of these mitochondria has as its basis an increased leakage of the primarily conserved energy.

MATERIALS AND METHODS

Animals and preparation of mitochondria

3–4 weeks old guinea pigs (Pir/Srr/c strain) were cold exposed at an environment of 5 °C for at least 6 days before sacrifice. This time of cold exposure has been shown to transform the brown adipose tissue mitochondria into a maximally loosely coupled state as judged by criteria discussed earlier[1]. Mitochondria from the interscapular brown adipose tissue from separate animals were prepared as described earlier[7].

Protein was determined using the Folin–Ciocalteu reagent[9].

Chemicals

Rotenone, oligomycin and nucleotides were the products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.). In some experiments ATP from P.L. Biochemicals (Milwaukee, Wisc., U.S.A.) was used. HEPES was obtained from Calbiochem (Luzern, Switzerland). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and atractyloside were gifts from P.G. Heytler of Du Pont, Wilm., U.S.A. and Dr R. Santi, University of Padua, Italy, respectively. [^{14}C]ADP was from The Radiochemical Center, Amersham, England. Other chemicals were of the highest purity commercially available.

Incubation of mitochondria

The mitochondria were incubated at 25 °C in a medium containing in a volume of 1 ml: 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer, pH 6.8, unless otherwise stated; 5 mM potassium phosphate buffer, pH 6.8; 135 mM sucrose; 2 mM EDTA; 1 mM $MgCl_2$; 3 μM rotenone and 3.3 mM KCN. The concentrations of ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

* The term "energy potential' is used synonymously with the term "energy pressure" as proposed by Slater and Welle[6] for the primarily conserved energy.

** *I.e.* cytochrome b_{561} + cytochrome $b_{565/558}$ (ref. 8).

(TMPD) were 4.2 mM and 90 μM, respectively. All acids were pH adjusted by KOH. Alterations and additions are indicated in the legends to figures and table.

Spectrophotometry

The measurement of oxidation–reduction level of *b*-type cytochromes were performed as previously described[7,8]. The temperature was thermostatically controlled at 25 °C.

Adenylate kinase activity

The mitochondria were suspended in the standard incubation medium (see above) at a concentration of 1.1 mg of protein per ml in the presence of ascorbate and TMPD. The reaction was started by adding 0.5 mM ADP containing 1 μCi [^{14}C]ADP. At different time intervals aliquots of 0.2 ml were extracted at −10 °C by an equal volume of 12% $HClO_4$. The extracts were further treated and the adenosine phosphates separated as described by Packham *et al.*[10] except that thin-layer chromatography on cellulose plates (MN-Polygram, Type Cel 300 from Macherey-Nagel and Co., Düren, Germany) was used instead of paper chromatography. The radioactivity was counted in a Packard Tri-Carb scintillation counter.

RESULTS

The relationship between phosphate potential and "energy potential"

When ADP was added to the mitochondria after ascorbate/TMPD (Fig. 1) cytochromes *b* were reduced, and this reduction was inhibited to 80–85% by oligomycin (*i.e.* the same degree of inhibition as that found with ATP[7]), and completely

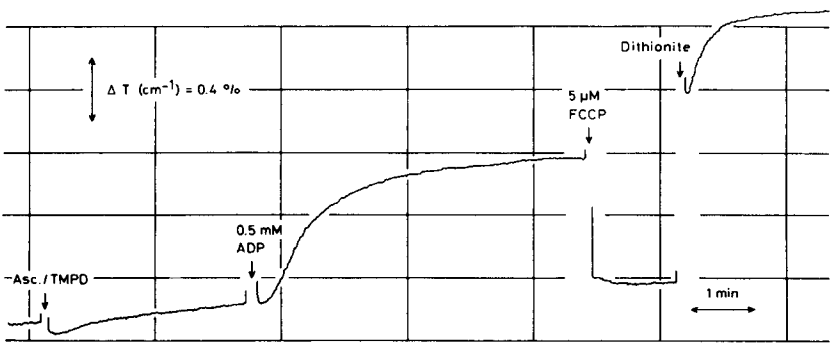

Fig. 1. The reduction of cytochromes *b* in brown adipose tissue mitochondria induced by ADP and the effect of FCCP. The mitochondria were suspended in the standard incubation medium, pH 6.06 (see Materials and Methods) at 0.7 mg of protein per ml. The change in transmission $\Delta T(\%) = \Delta(T_{564\text{ nm}} - T_{575\text{ nm}})$.

inhibited by FCCP. The response to ADP was, however, found to be more unstable upon prolonged storage of the mitochondria at 0 °C than that to ATP, and all experiments were carried out within 2–3 h after their preparation. From Fig. 2A it is seen that the extent of cytochromes *b* reduction reaches a maximum at almost the same concentration of ADP (approx. 0.7 mM) as that previously found for ATP (approx. 1.0 mM) (ref. 7) although the maximal "energy potential" obtained by ADP is lower

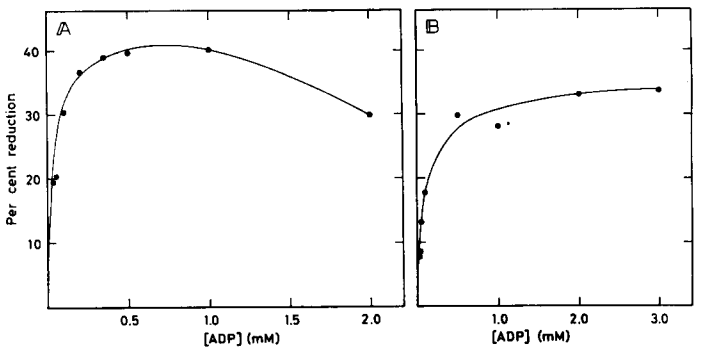

Fig. 2. Effect of ADP concentration on the reduction of cytochromes *b* in brown adipose tissue mitochondria (A) and liver mitochondria (B). The mitochondria were suspended in the standard incubation medium, pH 6.06 (A) and pH 6.8 (B) at 0.7 and 5.3 mg of protein per ml, respectively. Experimental procedure as described in Fig. 1, and the per cent reduction was measured at steady state. 100% reduction represents the difference in transmission, $\Delta(T_{564\ nm} - T_{575\ nm})$ between the level obtained by adding excess of dithionite and the initial ascorbate/TMPD level. Appropriate controls were taken to make corrections for the slow decrease in the ADP respons by time.

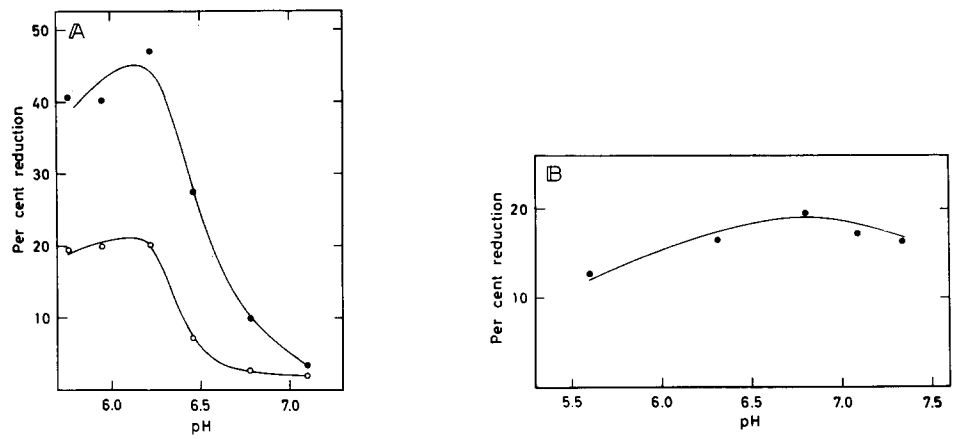

Fig. 3. Effect of pH on the ADP induced reduction of cytochromes *b* in brown adipose tissue mitochondria (A) and liver mitochondria (B). The mitochondria were suspended in the standard incubation medium at a concentration of 0.57 mg of protein per ml (A) and 6.0 mg of protein per ml (B). Experimental procedure as described in Fig. 1 at an ADP concentration of 0.5 mM, but with varying pH values. Per cent reduction was measured after 1 min (○—○) or at steady state (●—●). 100% reduction as defined in Fig. 2.

(*e.g.* see Fig. 4). The pH activity curve of the ADP induced reduction (Fig. 3A), although slightly displaced towards acid pH, was similar to that of ATP[7,8], but very different from that found with liver mitochondria (Fig. 3B).

Since the electron transport was inhibited by KCN in our *in vitro* system and the "energy potential" of the freshly isolated mitochondria is essentially zero[7], it was assumed that the oligomycin sensitive response to ADP was mediated *via* ATP generated by the adenylate kinase reaction. This conclusion was supported by results obtained by following the transformation of [^{14}C]ADP added to the reaction medium. The results (Table I) show that the adenylate reaction must be very active in brown

TABLE I

ATP FORMATION FROM EXOGENOUS ADP IN BROWN ADIPOSE TISSUE MITOCHONDRIA

The mitochondria were incubated in the standard incubation medium at pH values as indicated with 0.5 mM [^{14}C]ADP. The per cent distribution of the radioactivity was determined as described in Materials and Methods. Corrections were made for the nonenzymic contributions found to be 1.5% ATP and 1.5% AMP. The results represent the mean of three chromatographic runs.

	Per cent distribution of radioactivity							
	pH 7.4		*pH 6.8*		*pH 6.1*		*0.1 mM atractyloside pH 6.1*	
	13 s	*5 min*	*13 s*	*5 min*	*13 s*	*5 min*	*13 s*	*5 min*
ATP	7.0	11.8	8.5	15.0	8.6	15.4	6.4	17.7
ADP	82.9	73.2	84.0	62.7	82.7	53.9	87.6	55.0
AMP	10.1	15.0	7.5	22.3	8.7	30.7	6.0	27.3

adipose tissue mitochondria since up to half the added ADP was transformed to ATP+AMP at pH 6.1 during an incubation period of 5 min even at the low concentrations of free Mg^{2+} used in the assay. Atractyloside had only a minimal effect upon this transformation and it can therefore be assumed that this enzymic activity is mainly confined to the intermembrane space as in other types of mitochondria[11]. Further support for the involvement of adenylate kinase is the fact that AMP (Fig. 4) and fluoride (not shown) inhibit the ADP induced reduction whereas only a small inhibition of the ATP induced reduction was observed (Fig. 4). The adenylate kinase

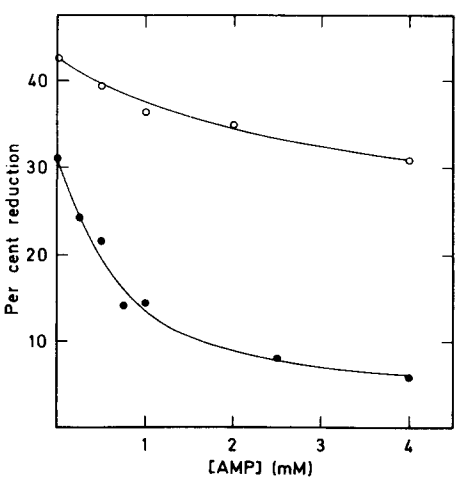

Fig. 4. Inhibition by AMP of the ADP (●—●) and ATP (○—○) induced reductions of cytochromes *b* in brown adipose tissue mitochondria. The experiments were conducted as in Fig. 1 after preincubation with AMP for 5 min at a mitochondrial protein concentration of 0.68 mg per ml. The pH was 6.2 (●—●) and 6.8 (○—○), and the concentrations of ADP and ATP were 0.5 and 1.0 mM, respectively. Per cent reduction was measured at steady state. 100% reduction as defined in Fig. 2.

activity may thus explain the effect of ADP on the tightening of coupling observed in brown adipose tissue mitochondria from both newborn[5] and cold-stressed guinea pigs[1] and its effect on the uptake of Ca^{2+} in these mitochondria[2].

When the extramitochondrial phosphate potential was lowered by addition of ADP to mitochondria maximally energized by ATP, a rapid but partial reoxidation of the cytochrome *b* complex was observed. Over the entire range of extramitochondrial phosphate potentials tested (Fig. 5) the per cent oxidation induced by ADP was higher in brown adipose tissue mitochondria than in liver mitochondria, the difference being most marked in the lower potential range.

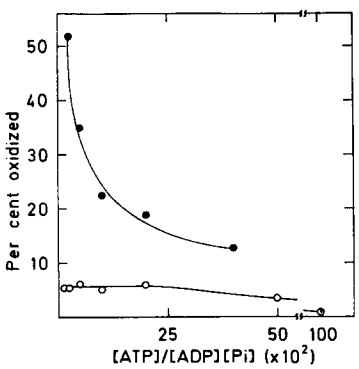

Fig. 5. Effect of ADP concentration on the ATP induced reduction of cytochromes *b* in brown adipose tissue mitochondria (●—●) and liver mitochondria (○—○). The mitochondria were suspended in the standard incubation medium, pH 6.8. ●—●, 0.5 mM ATP; 0.49 mg of mitochondrial protein per ml. ○—○, 0.25 mM ATP; 5.9 mg of mitochondrial protein per ml. The per cent oxidation is plotted as a function of the extramitochondrial phosphorylation potential after adding ADP at varying concentrations. 100% oxidation represents the difference in $\Delta T(\%) = \Delta(T_{564\text{ nm}} - T_{575\text{ nm}})$ values in the presence of ATP and 5 μM FCCP.

Effect of oligomycin on the generation and dissipation of the "energy potential"

The inhibition of respiration by oligomycin has been used as a major criterion for respiratory control[12] and as a measure of the intactness of mitochondria[13]. It is a general experience that when brown adipose tissue mitochondria are respiring on succinate or NADH-linked substrates in the absence of bovine serum albumin (and carnitine + ATP[14]), there is no inhibition of respiration by oligomycin. This lack of oligomycin inhibition may be due either to (1) a defect in the generation of an "energy potential" by operation of the respiratory chain, or to (2) a rapid dissipation of this primarily conserved energy, or to (3) a defect in the utilization for the synthesis of ATP of the "energy potential" *i.e.* a defective ATPase activity. In order to differentiate between these alternatives we have studied the effect of oligomycin on the "energy potential" generated by exogenous ATP.

In a previous study[7] it was found that oligomycin almost completely inhibits the ATP induced reduction of cytochromes *b*. Thus, there is no lack of ATPase activity in these mitochondria. Furthermore, upon addition of oligomycin to brown adipose tissue mitochondria energized by ATP (Fig. 6) cytochromes *b* become oxidized in a rather rapid reaction without any detectable lag period. In this reaction the rate of oxidation[15] as well as the steady-state redox level as compared to the

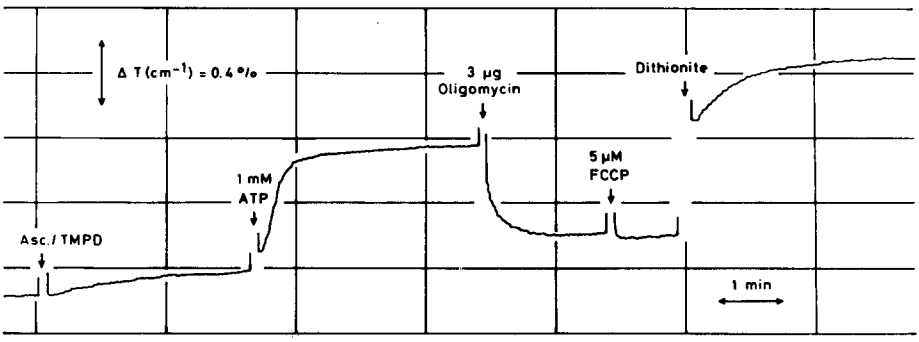

Fig. 6. Effect of oligomycin on the redox level of cytochromes *b* in brown adipose tissue mitochondria following maximal energization by ATP. The mitochondria were incubated in the standard incubation medium, pH 6.8, at 0.46 mg of protein per ml. The change in transmission, $\Delta T(\%) = \Delta(T_{564\text{ nm}} - T_{575\text{ nm}})$.

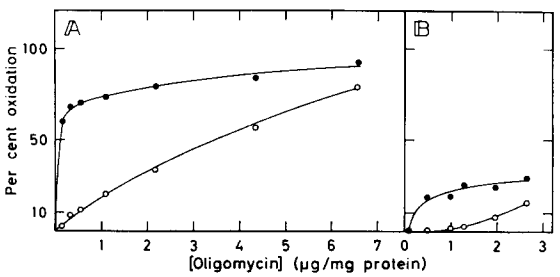

Fig. 7. Effect of oligomycin concentration on the rate, *i.e.* $t = 30$ s (○—○) and steady-state level (●—●) of oxidation of cytochromes *b* in brown adipose tissue mitochondria (A) and liver mitochondria (B) following maximal energization by ATP. The experiments were performed as described in Fig. 6 at mitochondrial protein concentrations of 0.46 mg per ml (A) and 7.63 mg per ml (B). 100% oxidation is defined in Fig. 5.

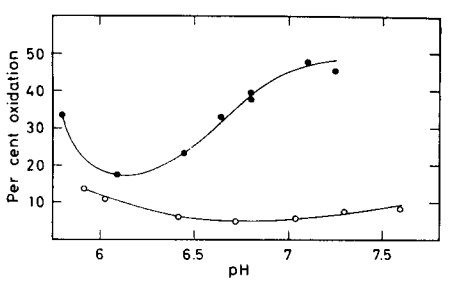

Fig. 8. Effect of pH on the rate of oxidation of cytochromes *b* induced by oligomycin in brown adipose tissue mitochondria (●—●) and liver mitochondria (○—○) following maximal energization by ATP. The experiments were performed as described in Fig. 6. 100% oxidation is defined in Fig. 5. ●—●, 0.45 mg of mitochondrial protein per ml; 1 µg of oligomycin; the rate is the degree of oxidation at 30 s. ○—○, 4.9 mg of mitochondrial protein per ml, 5 µg of oligomycin; the rate is the degree of oxidation at 60 s.

completely deenergized state (+FCCP), may be used to express the energy dissipation in quantitative terms. Fig. 7A shows the effect of different concentrations of oligomycin. It is seen that the initial rate of oxidation (% oxidized/30 s) as well as the

steady-state oxidation–reduction level is dependent on the concentration of added oligomycin up to about 7 μg per mg of protein, approaching an asymptotic value of approx. 85% steady-state oxidation of cytochromes *b*. The corresponding experimental curves for liver mitochondria are shown in Fig. 7B. It is clearly seen that the oligomycin sensitivity is considerably higher in brown adipose tissue mitochondria than in liver mitochondria and that the rate of oxidation, *i.e.* the rate of energy dissipation is approx. 15 times higher in brown adipose tissue than in liver mitochondria at pH 6.8. From Fig. 8 it is seen that the rate of energy dissipation is strongly pH dependent. Thus, with brown adipose tissue mitochondria a minimum of energy dissipation was obtained at pH 6.1 to 6.2. In contrast, in liver mitochondria the energy dissipation was much less pH dependent although an apparent pH minimum at pH 6.7 to 6.8 was obtained.

DISCUSSION

The mechanism of loose coupling in brown adipose tissue mitochondria has been the object of extensive investigations for the last few years with polarography as a principal tool. The limitations of such studies alone are obvious, however, since the energetic condition of the mitochondrial inner membrane can not be studied independently of the electron transport. More recently, the energy-dependent redox changes of the cytochrome *b* complex has been employed as an internal probe to study the factors affecting the generation of the "energy potential" in brown adipose tissue mitochondria more selectively[7,8]. In the present work these studies have been extended to include also the factors which affect the energy dissipation reaction.

Energization of brown adipose tissue mitochondria by ATP, as studied by the reduction of the cytochrome *b* complex, reveals two characteristic features different from that observed in other mitochondria so far studied. First, brown adipose tissue mitochondria require a high ATP concentration[7] (*i.e.* a high extramitochondrial phosphate potential) and the energization process shows a strong pH dependence[7,8]. Secondly, as shown in Fig. 5, the "energy potential" of these mitochondria is very sensitive to changes in the extramitochondrial phosphate potential, *e.g.* induced by ADP, in contrast to the situation in liver mitochondria of the same animals. This difference may at least partly be explained by the higher rate of energy dissipation in brown adipose tissue than in liver mitochondria (see below). The ADP-induced energization of both types of mitochondria revealed principally the same pattern of pH dependence as previously found for the ATP-induced energization[7], except that the pH activity curves with ADP are slightly displaced towards acid pH. This displacement is probably explained by the effect of pH on the adenylate kinase reaction (Table I and ref. 16).

It is a general experience that it is impossible to isolate mitochondria without so-called "leaks" of the "energy potential"[6]. The magnitude of the leakage has been estimated in brown adipose tissue and liver mitochondria maximally energized by ATP, from the rate and extent of the oligomycin-induced oxidation of the cytochrome *b* complex which reflects the dissipation of the "energy potential". Based on these criteria, it is seen from Figs 6 and 7 that this leakage is much more pronounced in brown adipose tissue mitochondria than in liver mitochondria, *e.g.* at pH 6.8 the energy dissipation is approx. 15 times higher. The pH dependence of this reaction is

a further indication of the importance of the transmembrane pH gradient in the control of coupling of electron transport to phosphorylation in brown adipose tissue mitochondria as earlier discussed[5,7,17,18].

In general, a leakage of the "energy potential" may be due to (1) structural changes of the mitochondria, (2) the presence of endogenous uncouplers, or (3) the utilization of energy for ion transport[6]. In the present study, strong experimental evidence is given in support of the view that the phosphorylation mechanism is well preserved in brown adipose tissue mitochondria as studied by their oligomycin- and FCCP-sensitive energization by ATP, and there is no evidence in support of the view that these mitochondria are uncoupled in the classical sense. On the other hand, evidence has been obtained that changes in the ultrastructure of brown adipose tissue mitochondria are related to the regulation of the degree of coupling by fatty acids[19]. If fatty acids exert their action in their dissociated form, then the observed effect of pH on the energy dissipation reaction (Fig. 8) could be explained.

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