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STUDIES ON THE ENERGY STATE OF ISOLATED BROWN ADIPOSE TISSUE MITOCHONDRIA

A COMPARATIVE STUDY OF COLD-STRESSED AND WARM-READAPTED GUINEA PIGS

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SUMMARY

1. Alterations occur in the energetic properties of brown adipose tissue mitochondria of guinea pigs on cold exposure.

2. Studies on the respiratory pattern of these mitochondria by oxygraphic methods revealed that those isolated from cold-stressed animals are more loosely coupled than those from warm-readapted animals. This difference in tightness of coupling could not, however, be correlated to differences in the content of mitochondrial cytochromes, flavoproteins or non-esterified fatty acids. Factors affecting the generation and dissipation of the "energy potential" have, therefore, been compared in the two types of mitochondria by using the redox state of the cytochrome *b* complex as an internal probe.

3. The energization induced by ascorbate and succinate was more pronounced in mitochondria from warm-readapted than from cold-stressed animals.

4. When energization was induced by ATP, the K_m' for the nucleotide was found to be 125 μM and 60 μM with mitochondria from cold-stressed and warm-readapted animals, respectively. Furthermore, the ATP-induced energization of mitochondria from warm-readapted animals was less stimulated by P_i and less pH dependent as compared to those from cold-stressed animals.

5. The rate of endogenous dissipation of the ATP-induced "energy potential", as measured by the rate of reoxidation of cytochromes *b* following oligomycin addition, was found to be markedly higher in mitochondria isolated from cold-stressed than from warm-readapted animals.

6. The significance of this difference in energy dissipation for the above findings and for the thermogenic function of brown adipose tissue mitochondria, is discussed.

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

INTRODUCTION

Studies on the energy metabolism of guinea pigs have revealed that the capacity for non-shivering thermogenesis is largely confined to brown adipose tissue and is most pronounced during the neonatal stage or during cold exposure of young animals[1]. Mitochondria isolated from brown adipose tissue of this species have disclosed a respiratory pattern characteristic of a loosening of coupling of phosphorylation to respiration which coincides with the transitions of the tissue into thermogenesis[2–5]. Furthermore, a number of factors such as fatty acids[6], adenine and guanine nucleotides[7–9], inorganic phosphate[10], and pH[3,9] have been observed to affect the respiratory control of isolated brown adipose tissue mitochondria, and it has been suggested that these factors may be involved in the regulation of the energy metabolism of these mitochondria *in vivo*. Such a view has gained some support from the observations that the degree of coupling of these mitochondria isolated from cold-stressed and warm-adapted young guinea pigs was positively correlated to the mitochondrial content of ATP and P_i, and negatively correlated to the amount of AMP[5]. No significant difference in the mitochondrial content of non-esterified fatty acids was, however, found in the two types of mitochondria[5]. If any of these factors were of significance as regulatory devices *in vivo*, a difference in their effect upon the energy metabolism would be expected in mitochondria isolated from either thermogenically active or non-active brown adipose tissue.

The mitochondrial cytochrome *b* complex has recently been shown to be a sensitive probe of the energy state of brown adipose tissue mitochondria isolated from cold-stressed guinea pigs[11,12]. This work has now been extended to a comparative study of factors affecting the generation and dissipation of the "energy potential"* in mitochondria isolated from either cold-stressed or warm-readapted animals. It is shown that the concentrations of ATP and P_i, and the pH of the medium affect the generation of the "energy potential" differently in the two types of mitochondria. Furthermore, it is shown that the rate of energy dissipation is considerably higher in mitochondria from the cold-stressed animals than from the controls. This fact supports our previous conclusion[12] that the basis for the loose coupling associated with thermogenesis of brown adipose tissue mitochondria is an increased dissipation of the primarily conserved energy.

MATERIALS AND METHODS

Animals and preparation of mitochondria

3–4-week-old guinea pigs (Pir/Srr/c strain) were cold exposed to an environment of 5 °C for at least 6 days before sacrifice. Another group of guinea pigs of the same age were retransferred to an environment of 22–23 °C after an identical cold stress, where they remained for at least 1 week before sacrifice. This special treatment of the animals was found convenient for obtaining brown adipose tissue mitochondria in a high yield from warm-readapted control animals[5].

Mitochondria from the interscapular brown adipose tissue from separate animals were prepared as earlier described[11]. Protein was determined using the Folin–Ciocalteu reagent[13].

* The expression "energy potential" is used as defined in ref. 12.

Chemicals

Rotenone, oligomycin and nucleotides were the products of the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) was obtained from Calbiochem (Luzern, Switzerland). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from P. G. Heytler of Du Pont, Wilm., U.S.A. Bovine serum albumin Fraction V, fatty acid free, "Pentex" was from Miles Laboratories Inc., Kankakee, Ill., U.S.A. Other chemicals were of the highest purity commercially available.

Incubation of mitochondria

The measurements of the oxidation–reduction level of *b*-type cytochromes were performed in cuvettes of 10-mm light path using an Aminco-Chance dual-wavelength spectrophotometer as earlier described[11,12]; for wavelength settings, see Results.

The mitochondria were incubated at 25 °C in a medium containing in a volume of 1 ml: 40 mM 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 added ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (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.

Oxygen uptake was measured polarographically[5] in the same medium as above (KCN omitted), but in the presence of 2% bovine serum albumin. The respiratory control ratio is defined as the ratio between the maximal rate of respiration in the presence of ADP (State 3) and the rate when the added ADP has been depleted (State 4)[14]. The ADP/O ratio was calculated according to Estabrook[15].

Analytical procedures

The mitochondrial contents of cytochromes and flavoproteins were estimated from the reduced (5 μM FCCP + dithionite) *minus* oxidized (5 μM FCCP) difference spectra obtained with a dual-wavelength spectrophotometer as described[16]. The following millimolar extinction coefficients (reduced *vs* oxidized) were used: flavoprotein, ε (465 nm − 510 nm) = 11.0[17]; cytochromes *b*, ε (564 nm − 575 nm) = 26.2[18]; cytochrome *c*, ε (550 nm − 540 nm) = 21.0[19]; cytochrome *a*, ε (605 nm − 618 nm) = 12.5[20].

The contents of non-esterified fatty acids in freshly isolated brown adipose tissue mitochondria from warm-readapted animals were determined as earlier described[5].

RESULTS

Respiratory patterns of brown adipose tissue mitochondria from cold-stressed and warm-readapted guinea pigs

Figs 1A and 1B show the characteristic difference in respiratory pattern between the two types of mitochondria when respiring on succinate (+rotenone) in a reaction medium containing 2% bovine serum albumin. Mitochondria from cold-stressed animals disclosed a sluggish response to the first addition of 0.1 mM ADP, followed by a slow transition into State 4 (Fig. 1A, Curve I). The second addition

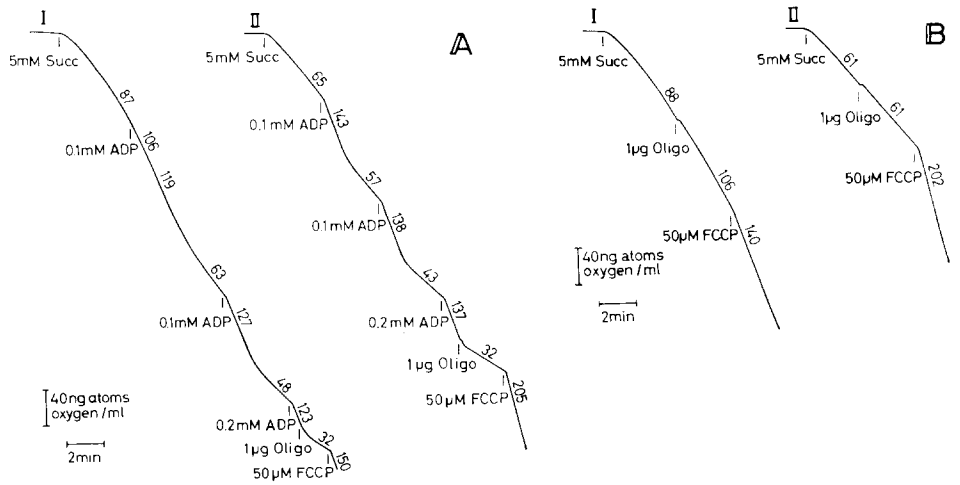

Fig. 1. Respiratory control experiments in brown adipose tissue mitochondria from cold-stressed (I) and warm-readapted (II) guinea pigs respiring on succinate (Succ) in the presence of 3 μM rotenone. The mitochondria (0.82 mg of protein) were suspended in 2 ml of the standard incubation medium, pH 6.8 (see Materials and Methods), containing 2% bovine serum albumin. (A) The respiratory responses to successive additions of ADP, oligomycin (Oligo) and FCCP. (B) The respiratory responses to additions of oligomycin (Oligo) and FCCP without prior energization by ADP[9, 12]. The numbers above each trace represent the specific respiratory rates (ngatoms O·min^{-1}·mg mitochondrial protein^{-1}).

of ADP resulted in a more normal stimulation of respiration into State 3. On the other hand, the first addition of 0.1 mM ADP to mitochondria of warm-readapted animals was followed by an immediate stimulation of respiration (Fig. 1A, Curve II). The respiratory control ratios with respect to the first addition of ADP were 1.89 and 2.51, respectively, for the two types of mitochondria, whereas the corresponding ADP/O ratios were 0.76 and 1.45, respectively. That this difference between the two types of mitochondria was not only apparent and due to an inhibition of the translocation of the added ADP[21, 22] is seen from Fig. 1B. The oligomycin-inhibited respiration was markedly less stimulated by FCCP with mitochondria from cold-stressed (Fig. 1B, Curve I) than with mitochondria from the warm readapted animals (Fig. 1B, Curve II). The ratio of the rate of respiration in the presence of FCCP to the rate in its absence was 1.32 in the former, and 3.3 in the latter case. Thus, both the ADP-released and the uncoupler-released control of respiration were markedly different in the two types of mitochondria showing that brown adipose tissue mitochondria from cold-stressed guinea pigs are more loosely coupled than those from warm-readapted animals.

The difference in the degree of coupling of the two types of mitochondria could not be correlated to any significant differences in the mitochondrial contents of either flavoproteins or cytochromes c ($+c_1$), or a ($+a_3$) per unit of mitochondrial protein (Table I). It should be noted, however, that the mitochondria of cold-stressed animals revealed a slightly, but significantly ($P<0.001$, Students t-test) lower content of cytochromes b reducible by dithionite. On the other hand, the content of cytochromes b reducible by ascorbate/TMPD upon energization by ATP were not significantly

TABLE I

MOLAR COMPOSITION OF THE RESPIRATORY CHAIN IN BROWN ADIPOSE TISSUE MITOCHONDRIA OF COLD-STRESSED AND WARM-READAPTED GUINEA PIGS

For experimental details, see Materials and Methods. The number of animals is included in the parentheses.

	Concentration (μmoles/g protein)	
	Cold-stressed (Mean ± S.D.)	*Warm-readapted (Mean ± S.D.)*
Flavoproteins*	6.32 ± 0.50 (7)	6.34 ± 1.05 (8)
Cytochromes *b***	1.29 ± 0.14 (7)	1.66 ± 0.20 (9)
Cytochromes *c* (+c_1)	2.70 ± 0.51 (7)	2.71 ± 0.29 (9)
Cytochrome *a****	1.54 ± 0.17 (7)	1.71 ± 0.21 (9)
$\frac{\text{Cytochromes } b}{\text{Cytochrome } a}$	0.84 (7)	0.97 (9)

* The absorbance changes at 465 nm − 510 nm is partly ascribed to reduction of the non-heme iron of different dehydrogenases[23].

** *I.e.* cytochrome b_{561} + cytochrome $b_{565/558}$ and both cytochromes contribute almost equally to the absorbance changes at 564 nm − 575 nm[11,12,24]. The concentration of cytochromes *b* in liver mitochondria from cold-stressed animals was found to be 0.18 ± 0.04 μmole per g protein ($n = 6$).

*** The absorbance changes at 605 nm − 618 nm is partly ascribed to reduction of the a_3 component of cytochrome *c* oxidase complex[20].

different (see below). This discrepancy can, however, not be explained at the moment. The values reported in Table I are all higher than those previously reported for brown adipose tissue mitochondria from unweaned guinea pigs[2] or cold-adapted hamsters[25]. This difference may at least partly be explained by the difference in methods used as well as in the selection of extinction coefficients. Furthermore, the Lowry method used for the assay of protein in the present study, gives somewhat lower values than the biuret method (Grav, H., unpublished) and may contribute in the same direction. In any case, the values are among the highest reported for animal tissues[26] using liver mitochondria (Table I) as a reference.

The contents of nonesterified fatty acids in freshly isolated brown adipose tissue mitochondria from warm readapted animals were 29.8 ± 6.2 nequiv per mg of protein ($n = 7$). Although the mean is somewhat lower it is within the ranges previously reported for brown adipose tissue mitochondria from either cold-stressed or warm-adapted guinea pigs[5].

The energy state of freshly isolated brown adipose tissue mitochondria and energization by substrate

We have previously shown that freshly isolated mitochondria from cold-stressed guinea pigs are completely deenergized, *i.e.* no oxidation of the cytochromes *b* occurs upon addition of FCCP to rotenone- and cyanide-inhibited mitochondria supplemented with ascorbate + TMPD at pH 6.8[11]. On the other hand, with liver mitochondria a 30% oxidation was seen under the same conditions[11] and with brown

adipose tissue mitochondria from warm-readapted animals a small but inconstant oxidation has occasionally been seen.

When succinate was added after ascorbate followed by FCCP it was apparent that a higher energy potential was created in mitochondria from the warm-readapted than from the cold-stressed animals. From Fig. 2 it is seen that this difference is pH dependent and most pronounced at the lowest pH range.

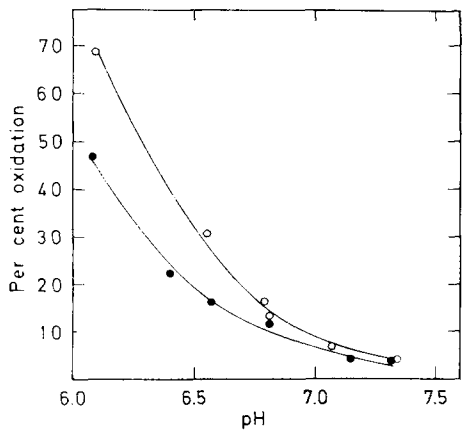

Fig. 2. Effect of pH on the substrate-induced energization of brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria were suspended in the standard incubation medium (see Materials and Methods) at 0.68 and 1.2 mg protein, respectively. To the mitochondrial suspension were added successively 4.2 mM ascorbate/90 μM TMPD, 5 mM succinate and 5 μM FCCP and the changes in transmission, Δ ($T_{564\ nm} - T_{575\ nm}$) was recorded. The FCCP-induced oxidation is given as per cent of the steady-state reduction induced by succinate.

Energization by ATP

When ATP is added to brown adipose tissue mitochondria inhibited with rotenone and cyanide and supplemented with ascorbate/TMPD, a reduction of cytochromes $b_{565/558}$ and b_{561} occurs[11,12,24]. This reduction is approx. 85% inhibited by oligomycin and completely inhibited by FCCP and thus energy dependent[12]. When the effect of ATP concentration upon this reduction was compared in the two types of mitochondria, it is seen from Fig. 3 that the per cent reduction obtained at saturating concentrations of ATP (as compared to total dithionite-reducible cytochromes *b*) is greater in the mitochondria from cold-stressed than from warm-readapted animals. With a large number of mitochondrial preparations it was found to be 46.9% $\pm$ 6.3 ($n=16$) in the cold-stressed and 31.0% $\pm$ 2.7 ($n=8$) in the warm-readapted animals. When these percentages are multiplied by the mean values for cytochromes *b* in Table I, they correspond to 0.61 and 0.51 μmole per g of protein, respectively, for the two types of mitochondria.

In addition, Fig. 3 shows that a higher concentration of ATP was needed to attain a maximal level of cytochromes *b* reduction (*i.e.* energization) with mitochondria from the cold-stressed animals ($K_m' = 125\ \mu$M) than those from the warm-readapted animals ($K_m' = 60\ \mu$M).

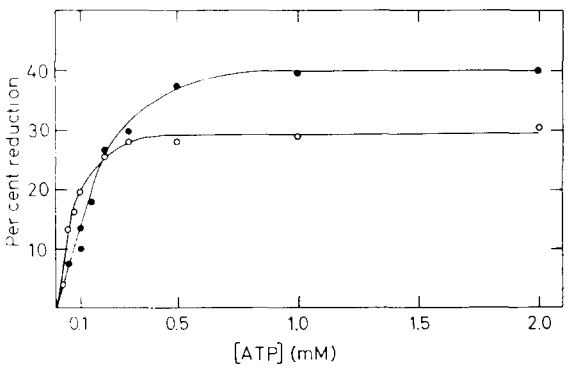

Fig. 3. Effect of ATP concentration on the reduction of cytochromes *b* in brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria (0.46 and 0.44 mg of protein, respectively) were suspended in 1 ml of the standard incubation medium, pH 6.8 (see Materials and Methods). ATP was added at varying concentrations and the per cent reduction measured at steady state. 100% reduction represents the difference in transmission, Δ ($T_{564\ nm} - T_{575\ nm}$) between the level obtained by adding excess of dithionite and the initial ascorbate/TMPD level. (For appropriate progress curve, see Fig. 5B of ref. 11.)

Effect of P_i

In brown adipose tissue mitochondria of cold-stressed guinea pigs P_i has a marked stimulatory effect on the ATP-induced energization[11]. This effect of P_i was found to be much smaller in mitochondria of the warm-readapted animals (Fig. 4).

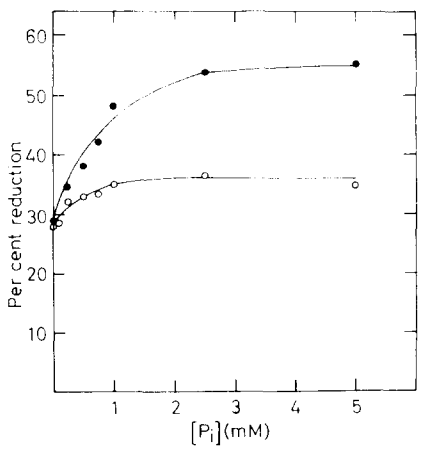

Fig. 4. Effect of P_i concentration on the ATP-induced reduction of cytochromes *b* in brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria (0.48 and 0.40 mg of protein, respectively) were suspended in 1 ml of the standard incubation medium, pH 6.8 (see Materials and Methods) except that the concentration of P_i was varied as shown. The reduction was induced by addition of 0.5 mM ATP and measured at steady state level. 100% reduction as defined in the legend to Fig. 3. (For appropriate progress curve, see Fig. 5B of ref. 11.)

Effect of pH

With both types of mitochondria the ATP-induced reduction is markedly influenced by the pH of the medium (Fig. 5). The effect is most pronounced in mitochondria of cold-exposed animals which require a more acidic pH to obtain maximal energization.

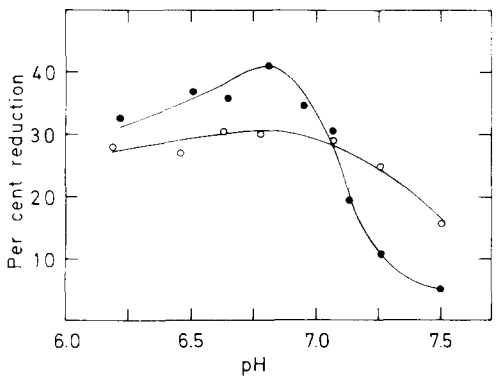

Fig. 5. Effect of pH on the ATP-induced reduction of cytochromes *b* in brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria (0.47 and 0.43 mg of protein, respectively) were suspended in 1 ml of the standard incubation medium (see Materials and Methods) except that the pH was varied as shown. The reduction was induced by addition of 1 mM ATP and measured at steady state. 100% reduction as defined in the legend to Fig. 3.

Rate of energy dissipation

When oligomycin is added to mitochondria maximally energized by ATP a concentration dependent oxidation of the cytochromes *b* is observed[12]. The rate of

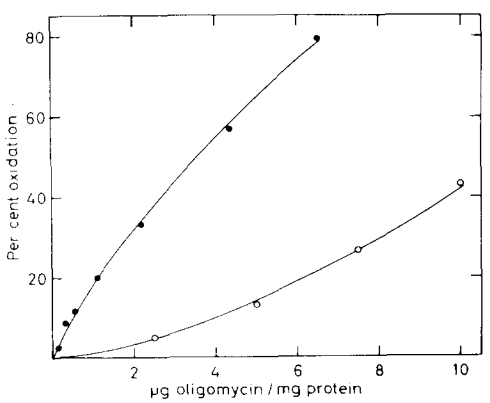

Fig. 6. Effect of oligomycin concentration on the redox level of cytochromes *b* following maximal energization by ATP in brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria (0.46 and 0.40 mg of protein, respectively) were suspended in 1 ml of the standard incubation medium, pH 6.8 (see Materials and Methods). 1 mM ATP was added followed by oligomycin after the steady-state level was reached. The per cent oxidation of cytochromes *b* was measured at 30 s. 100% oxidation represents the difference in the $\Delta T(\%)$ values in the presence of ATP and 5 μM FCCP. (For appropriate progress curve, see Fig. 6 of ref. 12.)

this oxidation is a reflexion of the rate of dissipation of the "energy potential", and we have previously found that this rate is markedly higher in brown adipose tissue than in liver mitochondria. In the present study the extent of oxidation after 30 s was used as an estimate of the rate of energy dissipation, and it was invariably found that the same amount of oligomycin produced a higher rate of dissipation in the mitochondria from cold-stressed than from control animals (Figs 6 and 7). The pH dependence of the energy dissipation reaction (Fig. 7) was essentially similar in the two types of mitochondria although a slight shift of the curve towards acidic pH was observed for the mitochondria of cold-exposed animals.

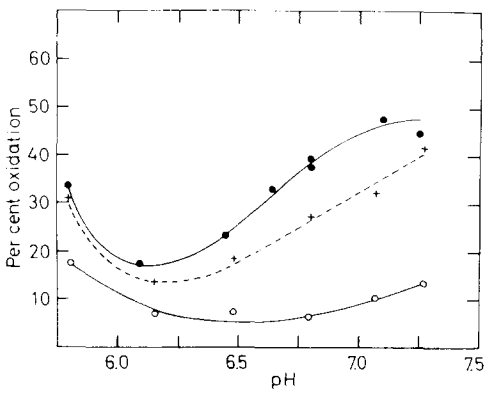

Fig. 7. Effect of pH on the oxidation of cytochromes *b* induced by oligomycin in brown adipose tissue mitochondria from cold-stressed (●—●) and warm-readapted (○—○) guinea pigs. The mitochondria (0.45 and 0.64 mg of protein, respectively) were suspended in the standard incubation medium (see Materials and Methods). The experiments were performed as described in the legend to Fig. 6 except that 1 μg of oligomycin was used and the pH was varied as shown. The per cent oxidation of cytochromes *b* was measured at 30 s. With brown adipose tissue mitochondria from warm-readapted animals the values at 60 s are also given (+---+).

DISCUSSION

On the basis of the known thermogenic function of brown adipose tissue[27], it is reasonable to assume that the mitochondria of cold-stressed guinea pigs are in a more active thermogenic state than those of warm-readapted animals[1]. This difference is clearly reflected in our *in vitro* respiratory control experiments (Fig. 1 and ref. 5) showing that mitochondria isolated from warm-readapted animals are more tightly coupled than those from cold-stressed animals. No control of respiration could, however, be detected in the absence of bovine serum albumin, and a further characterization of the energy transduction of the two types of mitochondria by oxygraphic methods alone has limited possibilities.

We have previously used the steady state redox level of the cytochrome *b* complex as a sensitive probe of the energy state of brown adipose tissue mitochondria from cold-stressed guinea pigs[11,12,24]. With these mitochondria it was found that the energy-dependent reduction of cytochromes *b* required a considerably higher ATP concentration than with liver mitochondria of the same animals[11]. Furthermore, exogenous P_i revealed a marked stimulatory effect on the ATP-induced reduction in

brown adipose tissue, but not in liver mitochondria, and the pH dependence of the reaction was very different in the two types of mitochondria[11]. Finally, the dissipation of the "energy potential" as measured by the rate of oxidation following addition of oligomycin to mitochondria maximally energized by ATP was approximately 15 times higher in brown adipose tissue than in liver mitochondria[12]. The present work has disclosed a higher stimulatory effect of P_i on the energization of brown adipose tissue mitochondria from cold-stressed than from warm-readapted animals (Fig. 4). This effect is similar to the higher stimulation by P_i on the energy-dependent uptake of Ca^{2+} in brown adipose tissue mitochondria of newborn or cold-stressed guinea pigs compared to those from 3-week-old warm-adapted animals[10] and may indicate a regulatory function of P_i in the energization process as previously discussed[11]. The content of P_i in mitochondria from warm-readapted animals was not found to be significantly higher than the values previously found in mitochondria from cold-stressed animals[5]. Thus, the values varied between 2 and 17 nmoles per mg mitochondrial protein ($n=7$), which indicated that the stimulatory effect is not directly related to the total content of endogenous P_i.

The difference in the pH dependence of the energization process in the two types of mitochondria (Fig. 5) strengthens our previous suggestion that the transmembrane pH gradient may be involved in the regulation of energy coupling in brown adipose tissue mitochondria. No correlation has been found between the total amount of free fatty acids and the degree of coupling in brown adipose tissue mitochondria. A small and probably specific fraction of endogenous fatty acids has, however, been shown to affect the coupling[6] and we have previously discussed the possibility that the pH dependence of the energy-linked reduction of cytochromes *b* in brown adipose tissue mitochondria may reflect the dissociation of fatty acids[12]. The difference in pH dependence of the two types of mitochondria (Fig. 5) may thus be explained by a larger amount of this specific fraction of fatty acids in the more loosely coupled mitochondria from the cold-stressed animals.

The higher concentration of ATP needed to obtain half maximal energization in mitochondria from the cold-stressed than from the warm-readapted animals (Fig. 3), is most easily explained by the higher rate of energy dissipation in these mitochondria (Figs 6 and 7). It is tempting to consider this difference in the rate of energy dissipation to be the bioenergetic basis of the difference in thermogenic activity of the two types of mitochondria (Fig. 1). This difference may therefore explain that the degree of loose coupling of brown adipose tissue mitochondria is primarily related to an increase in the resting respiratory rate (Fig. 1B).

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