BBA 46356

STUDIES ON THE ENERGY STATE OF ISOLATED BROWN ADIPOSE TISSUE MITOCHONDRIA

THE CYTOCHROME *b* COMPLEX AS A PROBE OF THE ENERGY STATE OF THE MITOCHONDRIAL INNER MEMBRANE

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(Received February 21st, 1972)

SUMMARY

Brown adipose tissue mitochondria from cold-stressed guinea pigs isolated in sucrose–HEPES–EDTA medium (HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) possess several criteria of loose coupling (J. I. Pedersen and H. J. Grav, *Eur. J. Biochem.*, 25 (1972) 75), and the present studies on the steady-state oxidation–reduction level of the cytochrome *b* complex under various experimental conditions have confirmed this conclusion. Generally, the kinetics as well as the steady-state redox level of *b*-type cytochromes in brown adipose tissue mitochondria differ markedly from those of coupled liver mitochondria of the same animals. The main features of these mitochondria are:

1. In the presence of rotenone, cyanide, ascorbate, and *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine (TMPD) the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) induces no oxidation of *b*-type cytochromes in brown adipose tissue mitochondria whereas a 30% oxidation was observed with liver mitochondria. This fact indicates that freshly isolated brown adipose tissue mitochondria are completely deenergized.

2. The percentage degree of reduction of b_{561} by succinate increases almost linearly from pH 6.0 (approx. 10%) to pH 8.0 (approx. 32%) in brown adipose tissue mitochondria, whereas the reduction of b_{561} in liver mitochondria (approx. 16%) is almost independent of pH in the same region.

3. With brown adipose tissue mitochondria, but not with liver mitochondria, the subsequent reduction of b_{565} by ATP was dependent on added P_i.

4. The ATP-induced reduction of b_{565} in liver mitochondria was completely inhibited by oligomycin whereas 87% inhibition was observed with brown adipose tissue mitochondria. The uncoupler FCCP, however, completely inhibited the ATP response. The oligomycin-insensitive reduction could also be induced by other high-energy nucleotides such as GDP and GTP.

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

5. The ATP-induced reduction of b_{565} in brown adipose tissue mitochondria revealed a pH dependence very different from that of liver mitochondria; the pH optium was around 7.4 in liver mitochondria whereas that of brown adipose tissue mitochondria was approx. 1 pH unit lower.

6. The concentration of ATP needed for half-maximal reduction of b_{565} was 30 times higher with brown adipose tissue mitochondria than with liver mitochondria under identical experimental conditions.

7. The significance of the pH and the intramitochondrial concentrations of adenine nucleotides and phosphate in the regulation of the coupling of respiration to phosphorylation in brown adipose tissue mitochondria is discussed.

INTRODUCTION

It is now well documented that brown adipose tissue is a major source of heat liberated by non-shivering thermogenesis (see ref. 1). Heat production derives from mitochondrial respiratory processes, the main substrates for which are free fatty acids released from the triglyceride stores by catecholamine stimulation (see refs. 2–4) mediated by cyclic AMP[5–7]. Increase in oxygen consumption is then concomitant with increased brown fat temperature. Strong evidence has been presented that the heat production is concomitant with a "loosening" of the coupling of mitochondrial oxidation to phosphorylation[8–10], a process found to have the physiological requirements of being temperature and time dependent as well as reversible[11]. In this "loosening" and "tightening" process it appears that at least two sets of factors are of importance, the mitochondrial free fatty acids[12] (or their acyl derivatives[13]) and the intramitochondrial pool of adenine nucleotides[11] and possibly also of guanine nucleotides[14]. The mechanism by which these elements regulate the coupling of phosphorylation to oxidation in these mitochondria has not yet been elucidated.

Recently it has been shown that the redox state of at least one of the cytochrome *b* species of the respiratory chain is dependent on the phosphorylation potential[15–18], and this makes it suitable as an internal probe of the energy state of the inner membrane[18]. In the present study this has been confirmed in brown adipose tissue mitochondria and the phenomenon has been used to characterize the conditions leading to energization of these mitochondria. It is concluded that the intramitochondrial concentrations of adenine nucleotides and P_i as well as the pH may constitute important parts in the mechanism regulating coupling of respiration to phosphorylation in brown adipose tissue mitochondria.

MATERIALS AND METHODS

Animals and preparation of mitochondria

Guinea pigs of the Pir/Srr/c strain were used. At an age of 3–4 weeks the animals were transferred from the breeding house to an environment of 5 °C where they remained 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[11].

The interscapular brown adipose tissue was excised and the mitochondria pre-

pared essentially as described earlier[19]. In order to remove trace amounts of erythrocytes interfering in the spectroscopic measurements, a slight modification was introduced. The tissue was homogenized in a medium consisting of 0.25 M sucrose, 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.2) and 0.2 mM EDTA, the volume was approximately 10 times the tissue weight. The homogenate was centrifuged at $4500 \times g_{av} \cdot \min$, $r_{av} = 9.4$ cm (Sorvall RC2-B centrifuge, SS 34 rotor) and the procedure was repeated once. After filtering through muslin the combined supernatants were centrifuged at $36500 \times g_{av} \cdot \min$ (SS 34 rotor). The mitochondrial pellet was resuspended in 10 ml of homogenization medium and centrifuged in a slightly conical Pyrex tube in the HB 4 swinging bucket rotor at $7100 \times g_{av} \cdot \min$, $r_{av} = 10.2$ cm. The erythrocyte-containing pellet was discarded and the supernatant centrifuged at $36500 \times g_{av} \cdot \min$ (SS 34 rotor). After an additional washing the final pellet was suspended in the homogenization medium at a mitochondrial protein concentration of 15–20 mg/ml. All preparative work was conducted at 0–2 °C. The mitochondria were prepared from separate animals unless otherwise stated. Reduced *minus* oxidized difference spectra revealed that erythrocytes (hemoglobin) had been completely removed.

When the mitochondria were tested in the standard incubation medium (KCN omitted, see below) no phosphate acceptor control was observed. However, when the medium was supplemented with bovine serum albumin (Pentex, fatty acid free) and either ATP, GDP, or GTP these brown adipose tissue mitochondria exhibited respiratory control (RC_{ADP}) with ratios between 2.5 and 3.5 with succinate (in the presence of rotenone) as substrate.

The stability of the mitochondrial preparation with respect to the parameters studied (see Results) was found to be satisfactory within a period of 10 h.

Liver mitochondria from the same animals were prepared in essentially the same way as the brown adipose tissue mitochondria.

Protein was determined using the Folin–Ciocalteu reagent[20].

Chemicals

Oligomycin, rotenone, ATP, GDP and GTP were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). In some experiments ATP produced by P.L. Biochemicals (Milwaukee, Wisc., U.S.A.) was used. HEPES (A grade) was purchased from Calbiochem (Luzern, Switzerland). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr P. G. Heytler of Du Pont. 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 HEPES buffer, pH 6.8 unless otherwise stated; 5 mM 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*-phenylene diamine (TMPD) were 4.2 mM and 90 μM, respectively. Alterations and other additions are indicated in text or legends to figures.

Spectrophotometry

The measurements of oxidation–reduction level of *b*-type cytochromes were performed in cuvettes of 10-mm light path using an Aminco-Chance dual-wavelength

spectrophotometer with both monochromators calibrated with reduced cytochrome *c* at an accuracy of ± 0.2 nm (for wavelength setting, see Results). The temperature was thermostatically controlled at 25 °C.

The spectra of the different *b*-type cytochromes were obtained by measuring the steady-state level of reduction with different settings of the measuring wavelength (575 nm as the reference). The spectra thus obtained were analyzed on an electronic curve analyzer (H. Hirschberg and T. Flatmark, unpublished results).

RESULTS

The spectral properties of the b-type cytochromes

KCN was added to the suspension of brown adipose tissue mitochondria to block the terminal part of the respiratory chain and rotenone was added to block the utilization of endogenous NADH-linked substrates. Furthermore, ascorbate and

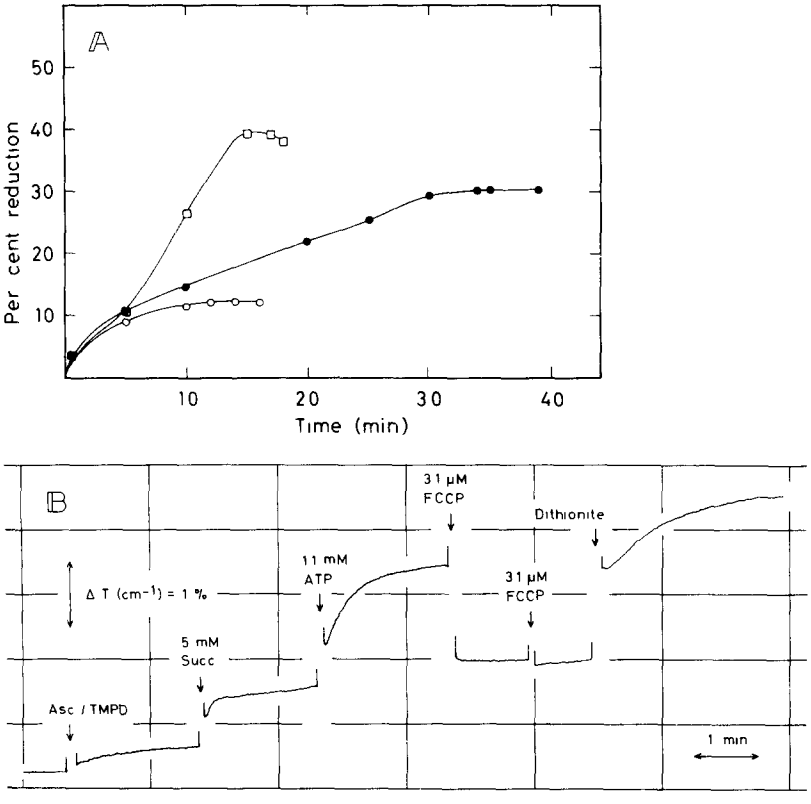

Fig. 1. (A) Partial reduction of cytochrome *b* of brown adipose tissue mitochondria by ascorbate + TMPD. The mitochondria were suspended in the standard incubation medium (see Methods) at 0.39 mg of protein per ml. The reaction was started by adding 4.2 mM ascorbate + 90 µM TMPD. 100% reduction represents the difference in transmission, $\Delta(T_{564\text{ nm}} - T_{575\text{ nm}})$ between the level obtained by adding excess dithionite and the initial ascorbate/TMPD level. □, pH 8.0; ●, pH 6.86; ○, pH 5.96. (B) The reduction of *b*-type cytochromes in brown adipose tissue mitochondria by ascorbate/TMPD, succinate and ATP and the effect of FCCP. The mitochondria (pooled sample of two animals) were suspended in the standard incubation medium (see Methods) at 1.58 mg per ml, pH 6.8. The change in transmission, $\Delta T(\%) = \Delta(T_{564\text{ nm}} - T_{575\text{ nm}})$

TMPD were added to reduce the cytochromes c_1, c and $a + a_3$ and to block the ATP responses in this part of the chain[21-23]. These additions did not change the redox state of the *b*-type cytochromes of liver mitochondria (Fig. 3D). In brown adipose tissue mitochrondria, however, a slow pH-dependent reduction was observed (Figs 1A and 1B). The species reduced was identified as a *b*-type cytochrome and an almost symmetrical spectrum with maximum around 561.5 nm was obtained. This "background" reduction by ascorbate/TMPD did not disappear upon the subsequent additions, *e.g.* of succinate and ATP and should be considered in all the progress curves shown in the following figures.

As expected, the addition of succinate to ascorbate/TMPD supplemented brown adipose tissue mitochondria (Fig. 1B) also resulted in reduction of a cytochrome *b* species with an absorption maximum around 561.5 nm (Fig. 2A). The reduction induced by the subsequent addition of ATP (Fig. 1B) resulted in an asymmetric cytochrome *b* spectrum (Fig. 2B) which could be resolved into two Gaussian curves with their maxima at 558.5 nm and 565 nm, respectively.

As seen from Fig. 1B the reduction of cytochrome b_{565} by ATP was reversed upon the addition of the uncoupler FCCP indicating its dependence on the "energy pressure". The absorbance change after addition of ATP is slightly greater than after addition of FCCP. This apparent discrepancy is totally explained by the "background" reduction of cytochrome b_{561} by ascorbate/TMPD (see above).

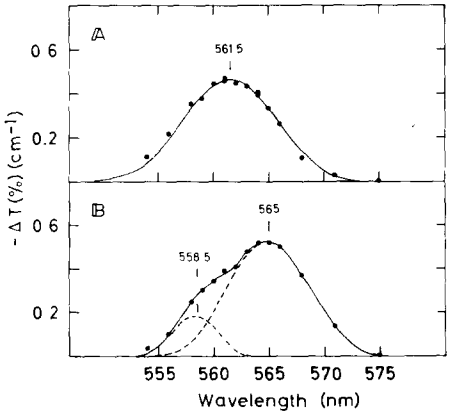

Fig. 2. The absorption spectra of *b*-type cytochromes of brown adipose tissue mitochondria reduced by succinate (A) and succinate + ATP (B) at 25 °C. The mitochondria were suspended in the standard incubation mixture (see Methods) at 0.65 mg of protein per ml. Succinate and ATP were added in sequence as shown in Fig. 1B and the steady-state change in transmission ($\Delta T(\%)$) was recorded with the reference wavelength set at 575 nm and the measuring wavelength as indicated on the abscissa. The experimental values fitted in (A) a single Gaussian curve (———) and in (B) a summation curve (———) of two Gaussian curves (– – – –).

ATP-induced reduction of cytochromes b

The effect of ATP on the redox state of the cytochrome *b* complex of brown adipose tissue mitochondria varies markedly with the *in vitro* conditions under which the reaction is studied. Firstly, the presence of Mg^{2+} in the incubation mixture is essential as it was found to stimulate the rate as well as the extent of the ATP induced transition, in agreement with the effect of this cation on oxidative phosphory-

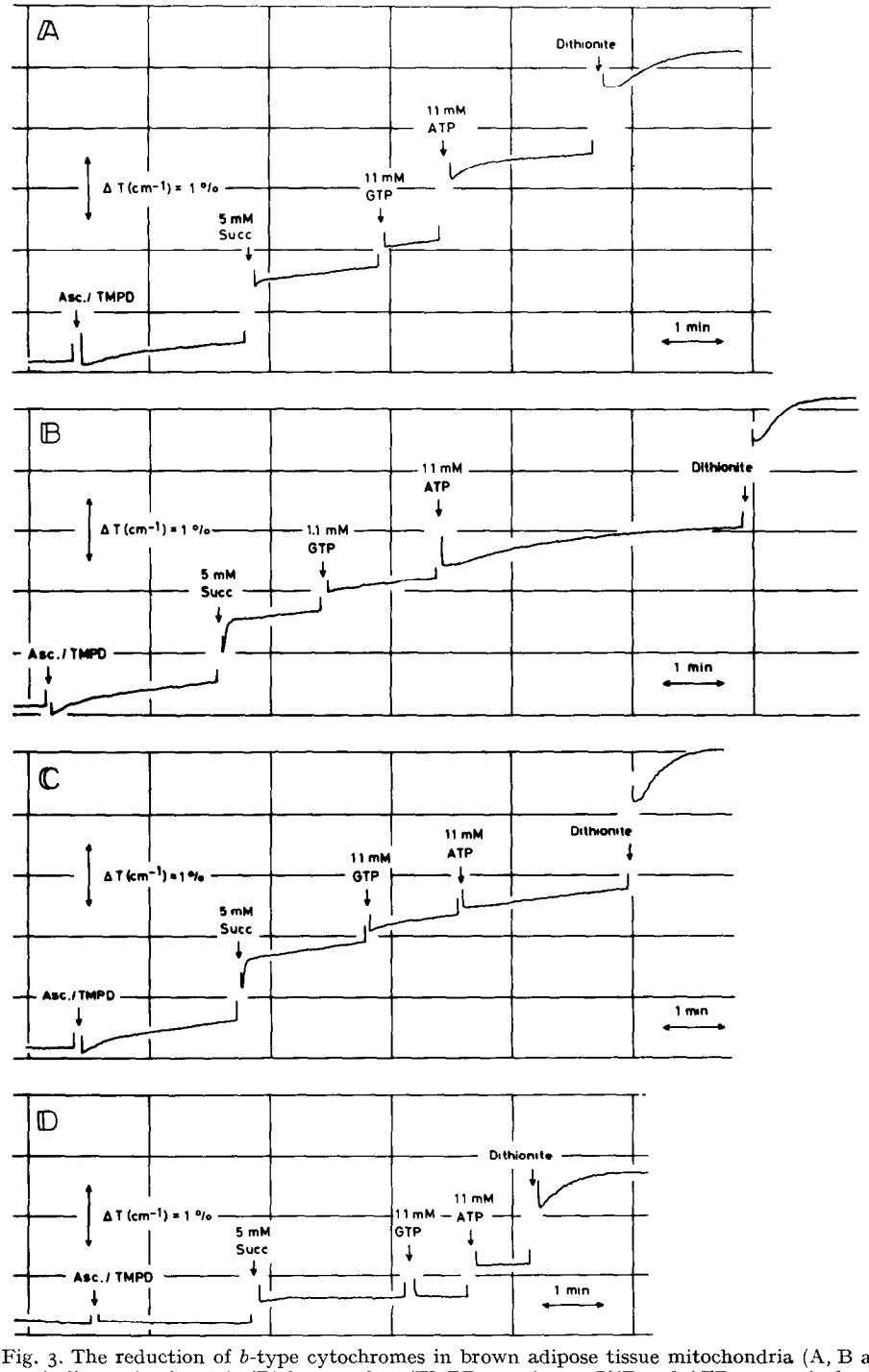

Fig. 3. The reduction of *b*-type cytochromes in brown adipose tissue mitochondria (A, B and C) and in liver mitochondria (D) by ascorbate/TMPD, succinate, GTP and ATP, respectively, in the presence of 5 mM P_i (A, C and D) and in the absence of added P_i (B). 2 μg oligomycin was present in (C). The mitochondria were suspended in the standard incubation medium, pH 6.8 (see Methods). (A, B and C) 2 mg of protein per ml (pooled sample of two animals). (D) 6 mg of protein per ml. The change in transmission $\Delta T(\%) = \Delta(T_{564\,nm} - T_{575\,nm})$.

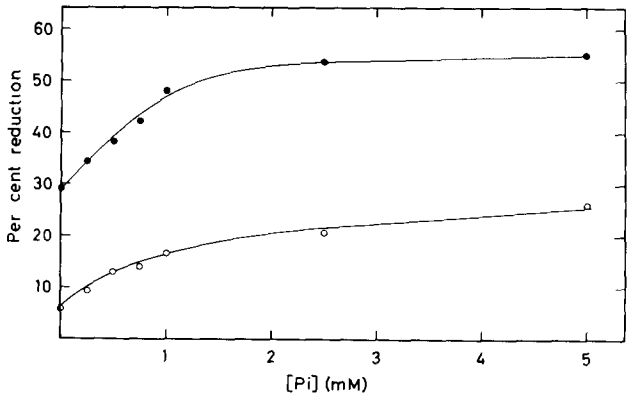

Fig. 4. The effect of P_i on the ATP-induced reduction of cytochrome *b* in brown adipose tissue mitochondria. The mitochondria (0.48 mg per ml) were suspended in the standard incubation medium, pH 6.8 (see Methods) except that the concentration of P_i was varied as shown. The reduction was induced by addition of 0.5 mM ATP (as shown in Fig. 5B), and the degree of reduction was measured at 30 s (○) and at steady state (●), respectively. 100% reduction as defined in legend to Fig. 1A.

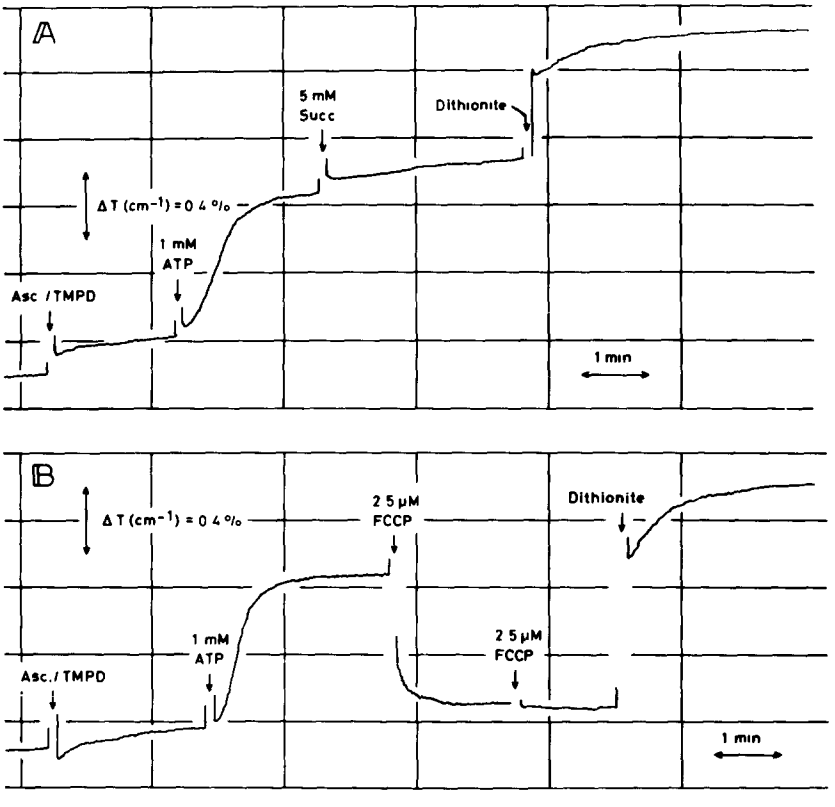

Fig. 5. The reduction of *b*-type cytochromes in brown adipose tissue mitochondria by ATP and succinate (A) and the effect of FCCP on the ATP-induced reduction (B). The mitochondria (0.63 mg of protein per ml) were suspended in the standard incubation medium, pH 6.8 (see Methods). The change in transmission, $\Delta T(\%) = \Delta(T_{564\,nm} - T_{575\,nm})$.

lation and repiratory control (RC_{ADP})previously observed with these mitochondria[19]. Secondly, we have repeatedly observed a rather pronounced stimulatory effect of P_i on the ATP-induced reduction of cytochrome b_{565} (followed by the wavelength pair 564 nm–575 nm), and a typical experiment is shown in Figs 3A and 3B. On the other hand, no such stimulation by this anion was observed with liver mitochondria from the same animals. The same dependence on exogenous P_i was observed in experiments where the energy-dependent reduction was induced without prior reduction of cytochrome b_{651} by succinate (Fig. 4).

When ATP was added to the mitochondria after ascorbate + TMPD (Fig. 5A) the absorbance difference between 564 nm and 575 nm, representing cytochromes b_{561} and b_{565}, showed, after a short lag period, a significantly higher increase than expected from the experiments reported above where the nucleotide was added after reduction of cytochrome b_{561} by succinate. From Fig. 5A it is also seen that the extent of the subsequent reduction by succinate was considerably reduced as compared to that observed when this substrate was added before ATP (Fig. 1B). As seen from Fig. 5B, however, the total reduction induced by ATP is sensitive to the uncoupler FCCP and thus energy dependent.

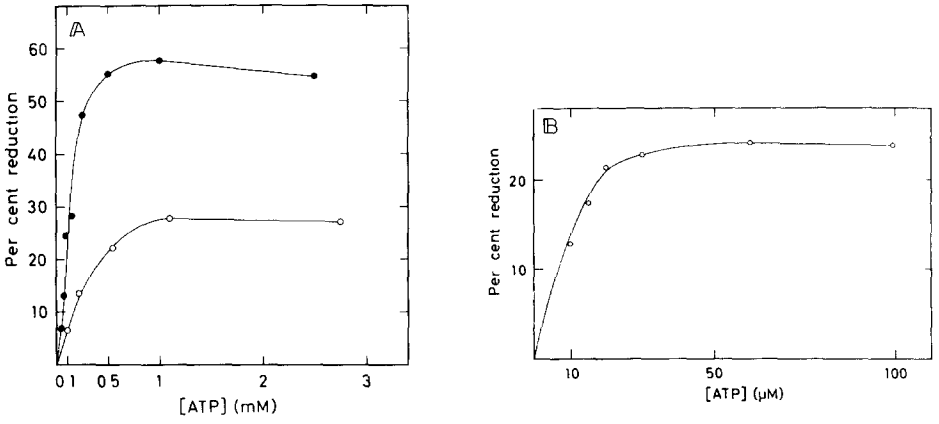

Fig 6. Effect of ATP concentration on the reduction of cytochromes *b* in brown adipose tissue mitochondria (A) and liver mitochondria (B) The mitochondria were suspended in the standard incubation medium (see Methods). ●—●, the reduction was induced by addition of ATP as shown in Fig. 5B; ○—○, the reduction was induced by addition of ATP after succinate as shown in Fig. 1B. 100% reduction as defined in legend to Fig. 1A.

Effect of ATP concentration

Figs 6A and 6B show the effect of different concentrations of ATP on the oxidation–reduction state of cytochromes *b* in brown adipose tissue and liver mitochondria. The ATP concentration needed to obtain half-maximal reduction of cytochrome b_{565} (ATP added after succinate) was 30 times higher in brown adipose tissue mitochondria (240 μM) than in liver mitochoncdria (8 μM). In brown adipose tissue mitochondria the ATP concentration needed for half-maximal reduction of cytochromes *b* decreased to 130 μM when the reduction was studied without prior addition of succinate.

Effect of pH on the succinate and ATP induced reductions of b-type cytochromes

By plotting the extent of reduction of *b*-type cytochromes, measured at 564

nm *minus* 575 nm as a function of pH, a marked difference was observed between brown adipose tissue and liver mitochrondria. With brown adipose tissue mitochondria the reduction of cytochrome b_{561} by succinate (Fig. 7A) increased with increasing pH whereas no such pH dependence was seen with liver mitochondria (Fig. 7B). The subsequent reduction of cytochrome b_{565} by the addition of ATP to brown adipose tissue mitochondria was strongly pH dependent with an apparent optimum around 6.5 (39% reduction). On the other hand, liver mitochondria (Fig. 7B) revealed a broad maximum in the range of pH 7.0 to 7.8 under the same conditions.

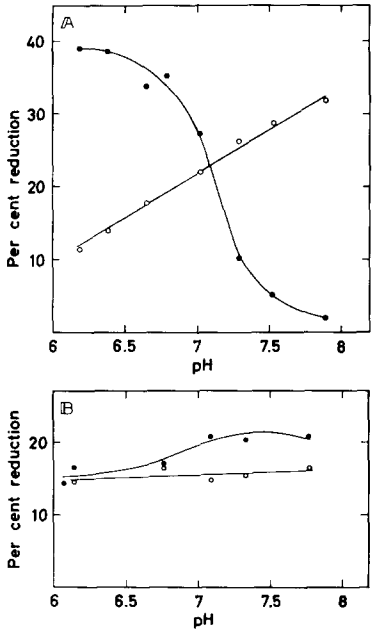

Fig. 7. Effect of pH on the reduction of *b*-type cytochromes induced by successive additions of succinate (○—○) and ATP (●—●) in brown adipose tissue mitochondria (A) and in liver mitochondria (B). For experimental details see Fig. 1 B. (A) 1.4 mg of mitochondrial protein per ml; succinate, 5 mM, ATP, 2.77 mM. (B) 5.5. mg of mitochondrial protein; succinate, 5 mM, ATP, 1 mM. 100% reduction as defined in legend to Fig. 1A.

Effects of inhibitors

When the uncoupler FCCP was added to freshly isolated mitochondria supplemented with ascorbate and TMPD, there was no oxidation of cytochromes *b* (as measured by the wavelength pair 564 nm–575 nm) in brown adipose tissue mitochondria (Fig. 8A) whereas a significant oxidation (approx. 30%) was observed in liver mitochrondria (Fig. 8B). This result reflects a difference in the "energy state" of the two types of mitochondria; the brown adipose tissue mitochondria being completely deenergized whereas the liver mitochondria are characterized by the presence of a certain "energy pressure". This difference in "energy pressure" of the two types of mitochondria is probably related to the very low concentration of endogenous ATP observed in freshly isolated brown adipose tissue mitochondria[11] as compared to that in liver mitochondria [24].

The inhibitor of oxidative phosphorylation, oligomycin, almost completely

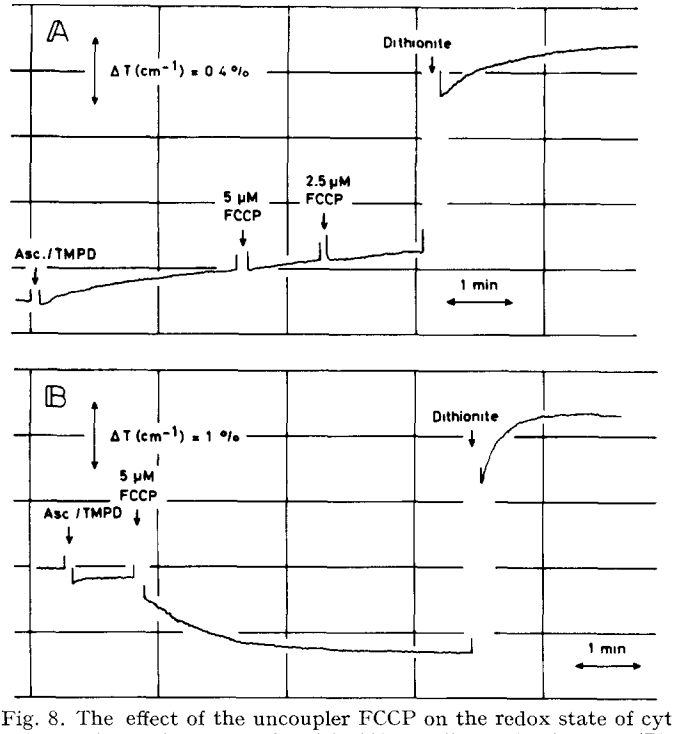

Fig. 8. The effect of the uncoupler FCCP on the redox state of cytochrome *b* in freshly isolated brown adipose tissue mitochondria (A) and liver mitochondria (B). The mitochondria were suspended in the standard incubation medium (see Methods) at a protein concentration of 0.5 mg per ml (A) and 4.9 mg per ml (B) The change in transmission. $\Delta T(\%) = \Delta(T_{564\,nm} - T_{575\,nm})$.

abolished the ATP-induced reduction of cytochrome b_{565} (Fig. 3C, see also Fig. 9). At a concentration of 3.5 μg oligomycin per mg of protein and at pH 6.8 the inhibition was 87%. Complete inhibition of this reduction was obtained with the uncoupler FCCP (Figs 1B and 5B). On the other hand, in liver mitochondria oligomycin at the same concentration completely inhibited energization by exogenous ATP. Thus, there seems no doubt that there are two types or mechanisms for the ATP-induced energization of brown adipose tissue mitochondria where the main contribution is primarily dependent on the function of ATP in the oxidative phosphorylation mechanism (see below and Discussion).

Effect of other high-energy nucleotides

It has previously been shown that high-energy nucleotides other than ATP have a stimulatory effect upon the respiratory control by ADP in different types of brown adipose tissue mitochondria[8,25]. Similarly, GTP and GDP were found to produce a small but significant pH-dependent reduction of the cytochrome *b* complex in brown adipose tissue mitochondria (Figs 3A, 3B and 9) whereas no such effect was observed with liver mitochondria (Fig. 3D). The GTP/GDP-induced reduction did not require the presence of P_i (Fig. 3B) and was found to be insensitive to oligomycin (Fig. 3C), but completely abolished by the uncoupler FCCP. The response was concentration dependent with a saturating concentration somewhat lower for GDP (approx. 1 mM) than for GTP (approx. 5 mM).

The extent of the GDP- and GTP-induced reduction corresponded to the oligomycin-independent reduction by ATP and is a further indication of two different types of energization of brown adipose tissue mitochondria (Fig. 9).

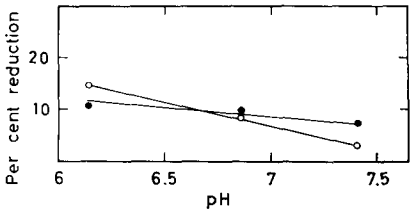

Fig. 9. Effect of pH on the reduction of cytochrome *b* induced by GTP (○—○) and ATP in the presence of 1 μg oligomycin (●—●). The mitochondria were suspended in the standard incubation medium (see Methods) at 0.63 mg of protein per ml. Ascorbate/TMPD was added prior to the nucleotides which were added at a concentration of 1 mM (ATP) and 2 mM (GTP). 100% reduction as defined in legend to Fig. 1A.

DISCUSSION

A direct role for cytochrome *b* in mitochondrial electron transport and energy conservation has been postulated[15,23,26-28]. Oxidation–reduction titrations have revealed the existence of at least two distinct species of *b* cytochrome in rat liver[29] and pigeon heart[30] mitochondria, one of which has an energy-dependent oxidation–reduction midpoint potential [29]. At room temperature one of these cytochromes has a single symmetric α band at 561 nm (termed b_{561}) and the other one has a double α band at 565 nm and 558 nm (termed b_{565})[16,17]. In intact pigeon heart mitochondria cytochrome b_{561} is readily reduced by succinate in both coupled and uncoupled mitochondria whereas cytochrome b_{565} is readily reduced by succinate in the presence of ATP but in uncoupled mitochondria[16,17].

In the present studies of brown adipose tissue mitochondria from cold-stressed quinea pigs we have observed the same species of cytochrome *b* as described in pigeon heart and rat liver mitochondria. Of particular interest is the demonstration of an energy (ATP)-dependent reduction of the cytochrome b_{565} species which is sensitive to the inhibitor oligomycin as well as to the uncoupler FCCP. In oxygen uptake studies with brown adipose tissue mitochondria such sensitivities have previously not been observed under similar conditions *in vitro*. The behaviour of the steady-state oxidation–reduction level of the *b*-type cytochromes of brown adipose tissue mitochondria as well as the kinetics differ, however, in several ways from those of liver mitochondria. These differences may be related to the specialized (thermogenic) function of these mitochondria.

It has been stressed by Pedersen and Grav[11] that brown adipose tissue mitochondria appear to exist in a fundamentally loosely coupled state, and the present data adds further support to this view. The complete lack of FCCP response to the redox state of cytochromes *b* (Fig. 8A) indicates that freshly isolated brown adipose tissue mitochondria are completely deenergized in contrast to the liver mitochondria (Fig. 8B). Thus, the low level of endogenous ATP in these mitochondria[11] is insufficient to energize the inner membrane.

The rather dramatic effect of P_i on the ATP-induced and FCCP-sensitive reduc-

tion of cytochromes *b* in brown adipose tissue mitochondria (characteristic of an energization) (Figs 3 and 4) as compared to the lack of response in liver mitochondria points to the interpretation that a deficiency in phosphate may play a major role in the mechanism of the loose-coupling in brown adipose tissue mitochondria. This conclusion is in accordance with Pedersen and Grav[11] demonstrating that these mitochondria are deficient in endogenous P_i and is also supported by the fact that P_i is required for energy-dependent Ca^{2+} uptake in brown adipose tissue mitochondria[31]. That some reduction is seen also in the absence of added P_i may be partly explained by a small amount of endogenous P_i, but in addition some contamination of ATP by P_i as well as the "unspecific" oligomycin-insensitive reduction (see Fig. 9) may contribute to this effect.

In heart and liver mitochondria P_i has, besides its obvious role in oxidative phosphorylation, marked effect on the energy-dependent uptake of cations[32-34]. More recently Papa *et al.*[35] have presented evidence that the addition of P_i to P_i-depleted liver mitochondria, under conditions of inhibition of oxidative phosphorylation, affects the "energy pressure" established by the oxidoreductions of the components of the respiratory chain. An effect of P_i on the translocation of ATP should also be seriously considered since it has been shown that inhibition of P_i transport also inhibits the ADP/ATP exchange in rat liver mitochondria[36].

The oligomycin-sensitive energization induced by ATP certainly involves several steps. The significantly lower initial rate of this reduction in brown adipose tissue mitochondria as compared to liver mitochondria and the much higher ATP concentration required (*cf.* Figs 7A and 7B) may be explained either by an inhibition of the translocation of adenine nucleotides, by a low ATPase activity[37] or by an increased leakage of the "energy pressure". As the initial rate of energization is more affected than the steady-state level it may be suggested that the translocation of ATP is inhibited. Under *in vitro* conditions similar to those used in the present work, the rate of ATP translocation has in fact been found to be lower in brown adipose tissue than in liver mitochondria when expressed per mg of mitochondrial protein[38].

The increased rate and extent of the oligomycin-sensitive reduction of cytochrome b_{565} by ATP at decreasing pH values (Fig. 7A) is in contrast to the situation in liver mitochondria (Fig. 7B) of the same animals. This pH dependency is almost identical to the effect of pH on oxidative phosphorylation and respiratory control in brown adipose tissue mitochondria from guinea pigs[19,25], rats and hedghogs[39]. Recent studies (T. Flatmark and J. I. Pedersen, unpublished results) undertaken to determine in more quantitative terms the relative significance of the reactions determining the "energy pressure" have shown that the rate of energy dissipation is much higher in brown adipose tissue than in liver mitochondria. Furthermore, this energy dissipation has its minimum at a low pH value (approx. 6.1). In addition, the redox potentials of cytochromes *b* and their dependence on pH[40] as well as their accessibility may differ in the two types of mitochondria. Such differences may explain the reduction of a cytochrome b_{561} species by ascorbate/TMPD in brown adipose tissue mitochondria (Figs 1A and 1B) not seen in the liver mitochondria (Fig. 3D), and also why the succinate-induced reduction of cytochrome b_{561} in brown adipose tissue mitochondria is more influenced by pH (Fig. 7A) than in liver mitochondria (Fig 7B).

The percentage reduction of the cytochrome *b* complex of brown adipose tissue mitochondria is nearly twice as high when ATP is added after ascorbate/TMPD as

compared to when it is added after succinate (Fig. 6A). The explanation for this is that in the absence of succinate ATP induces a reduction not only of cytochrome b_{565} but also of cytochrome b_{561} (T. Flatmark and J. I. Pedersen, unpublished results).

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