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METABOLIC CONSEQUENCES OF LIMITED SUBSTRATE ANION PERMEABILITY IN BROWN FAT MITOCHONDRIA FROM A HIBERNATOR, THE GOLDEN HAMSTER

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Brown fat mitochondria obtained from a hibernator, the golden hamster, were investigated in order to elucidate the significance of membrane permeability for metabolic functioning at different temperatures. The mitochondria were shown to have active permeases for phosphate and pyruvate, but very poorly developed permeases for di- and tricarboxylate substrate anions. This was shown with both osmotic swelling techniques and respiration-driven uptake studies. It was shown that the very limited malate permeation observed was compatible with it being a non-carrier-mediated diffusion process. The role of malate transport in supporting fatty-acid oxidation in vitro as a function of temperature was studied in detail. The results support our earlier suggestion that physiologically pyruvate carboxylase probably functions to generate oxaloacetate when high concentrations of condensing partner are needed during thermogenesis. They may also explain earlier observations that acetate was produced from palmitoyl-carnitine at low temperatures even when malate was present; this is here shown to be due to the limited malate permeability at these low temperatures. Thus, even at the body temperature of the hibernating hamster (4–5°C), brown fat is probably able to combust fatty acids totally.

Introduction

Mitochondria from brown adipose tissue are highly specialised for the rapid oxidation of fatty acids [1–3], and, in consequence, require a source of oxaloacetate in order to provide a condensing partner for acetyl-CoA coming from β-oxidation during the high rates of fatty-acid oxidation which occur in thermogenesis [4]. In an earlier investigation of brown fat mitochondria from hamster, we demonstrated a highly active pyruvate carboxylase and suggested that physiologically these mitochondria could use pyruvate in order to generate the requisite oxaloacetate to support fatty acid oxidation [5]. However, in in-vitro studies of these mitochondria, malate is often used for convenience [6–9].

Hamsters are hibernators whose body temperature during hibernation drops close to zero, so that an investigation of the influence of temperature on metabolic activity has physiological significance. In the present study, we have examined the pattern of substrate permease activities in hamster brown fat mitochondria, especially the permeases for the oxaloacetate precursors malate and pyruvate, and their functioning at hibernation temperatures. Particular emphasis has been placed on the role of malate permeation for supporting fatty-acid

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Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulfonic acid; EC_{50}, effective concentration yielding half-maximum response; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

oxidation at various temperatures. The results corroborate the suggested physiological role of pyruvate carboxylase in supporting fatty acid oxidation, since di- and tricarboxylic acids are almost not transported across the mitochondrial inner membrane, whereas pyruvate is readily transported. The observations may also explain earlier results on the acetate production from fatty acids which occurs in vitro at low temperatures [7]. Finally, they may in part explain the inability of hamster brown fat to show high rates of fatty acid synthesis.

Methods

Animals

Adult golden hamsters (*Mesocricetus auratus*) of both sexes were used for the preparation of brown fat mitochondria. They had been acclimated to cold (5 ± 1°C) for at least three weeks on an 8–16 h light-dark cycle. They had free access to food (a rabbit chow/sunflower-seed-based diet) and water. Adult rats of the Sprague-Dawley strain of both sexes were kept at 22 ± 2°C on a 12–12 h light-dark cycle, and had free access to chow (R3, Ewos, Sweden) and water, but were starved overnight before they were killed.

Mitochondria

Mitochondria were isolated from the pooled interscapular, cervical and axillary brown fat from 1–2 hamsters, as previously described [1]. The isolation medium was 250 mM sucrose. For respiratory studies the final wash and suspension of mitochondria were in 100 mM KCl, 20 mM K-Tes (pH 7.2). Liver mitochondria were prepared by conventional methods [10] and suspended in 250 mM sucrose. Protein concentration was determined by the biuret method.

Determination of substrate permeability

Determination by osmotic swelling techniques. Substrate permeability was measured essentially as described by Chappell and Crofts [11]. 100 mM solutions of the ammonium salts of the substrate anions, buffered with NH_4-Tes (10 mM) to pH 7.2 were used. Where necessary, 3 mM of the supporting anions were present initially. In addition, the medium contained 3 μM rotenone and 0.5 μg/ml antimycin A. 0.3 mg mitochondrial protein was used per ml. The total volume was 3 ml and the temperature routinely 23°C. The change in absorbance at 520 nm with time was determined. A decrease in absorbance was taken to indicate mitochondrial swelling caused by anion uptake. When studies were performed at different temperatures, the temperature in the cuvette was controlled by Peltier elements sitting in close contact with the sides of the cuvette. The temperature in the solution was checked with a thermistor probe at the beginning and end of each trace. The driving force for uptake was maintained constant under all conditions. This resulted naturally in different osmotic conditions for the different anions. Control experiments with all solutions at 200 mOsM gave qualitatively similar results to those reported here (not shown).

Determination by respiration-driven uptake. Respiration-driven substrate uptake was determined essentially as described by Halestrap and Denton [12], using ^{14}C-labelled substrate anions, and [6,6′(n)-^{3}H]sucrose as a marker of the extramitochondrial volume. The medium was 100 mM KCl, 20 mM K-Tes and 5 μM rotenone (pH 7.2). Succinate (2.5 mM) was used as respiratory substrate with rat liver mitochondria and glycerol-3-phosphate (5 mM) with brown fat mitochondria. 3 mM phosphate and malate were present where required. In addition, when brown fat mitochondria were studied, 2 mM GDP and 1% fatty-acid-free bovine serum albumin were also present in order to transfer the mitochondria into an energy-conserving state [1]. 8 mg mitochondrial protein in 1 ml medium were preincubated at 5°C for 5 min in the presence of about 50000 cpm ^{3}H-labelled sucrose. At zero time the ^{14}C-labelled anion was added (not under 50000 cpm) at concentrations of 50 or 150 μM. At the times indicated, the reaction was stopped by centrifugation for 3 min at 5°C in a rapid, table-top centrifuge ($g_{max} = 7000 \times g$) (Wifug, Stockholm). The tube was inverted, the walls rinsed with 2×0.5 ml incubation medium, and the pellet suspended in water, quantitatively transferred to a scintillation vial and counted in 5 ml toluene/Triton X-100 (7 : 3, v/v) plus 0.5% diphenyloxazole (PPO) in an Intertechnique liquid scintillation spectrometer.

Pyruvate carboxylation

Pyruvate carboxylation was measured as earlier described [5] by [^{14}C]HCO_3^- fixation.

Respiratory studies

Respiration studies were performed in an oxygen electrode chamber of 1 ml volume which contained a Clark-type Yellow-Springs oxygen probe 4004. The temperature in the chamber was controlled by a circulation waterbath, and the temperature of the medium in the chamber was measured with a thermistor probe at the beginning and end of every trace. The medium contained 100 mM KCl/20 mM K-Tes/2 mM $MgCl_2$/4 mM K-phosphate/1 mM EDTA (pH 7.2). The concentration of mitochondrial protein was 0.7 mg/ml. Concentrations of malate, varying from 0 to 35 mM, were present initially as indicated. The reaction was started by the addition of a defined amount of a stock palmitoyl-L-carnitine solution. For each temperature, the amount used was that which, at 35 mM malate, resulted in the utilization of about 70% of the available oxygen. The amount of oxygen consumed and the maximal rate of oxidation was determined for each temperature and malate concentration. The oxygen content of the medium was taken to be 738, 628, 536, 474 and 434 nmol O per ml at 8, 15, 23, 30 and 37°C, respectively [13].

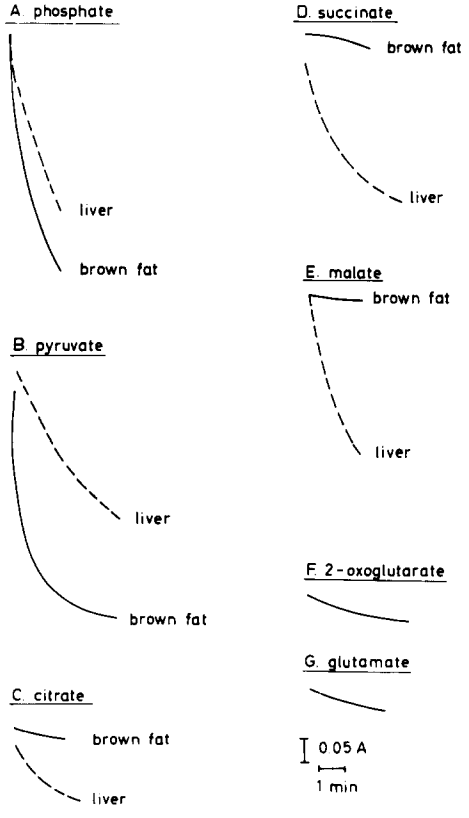


Fig. 1. Substrate anion uptake in hamster brown fat mitochondria, as studied in isoosmotic swelling experiments (see Methods). Traces with rat liver mitochondria are included for comparison (– – –). For succinate and malate, phosphate was present as counter ion; for citrate and oxoglutarate, both phosphate and malate were present.

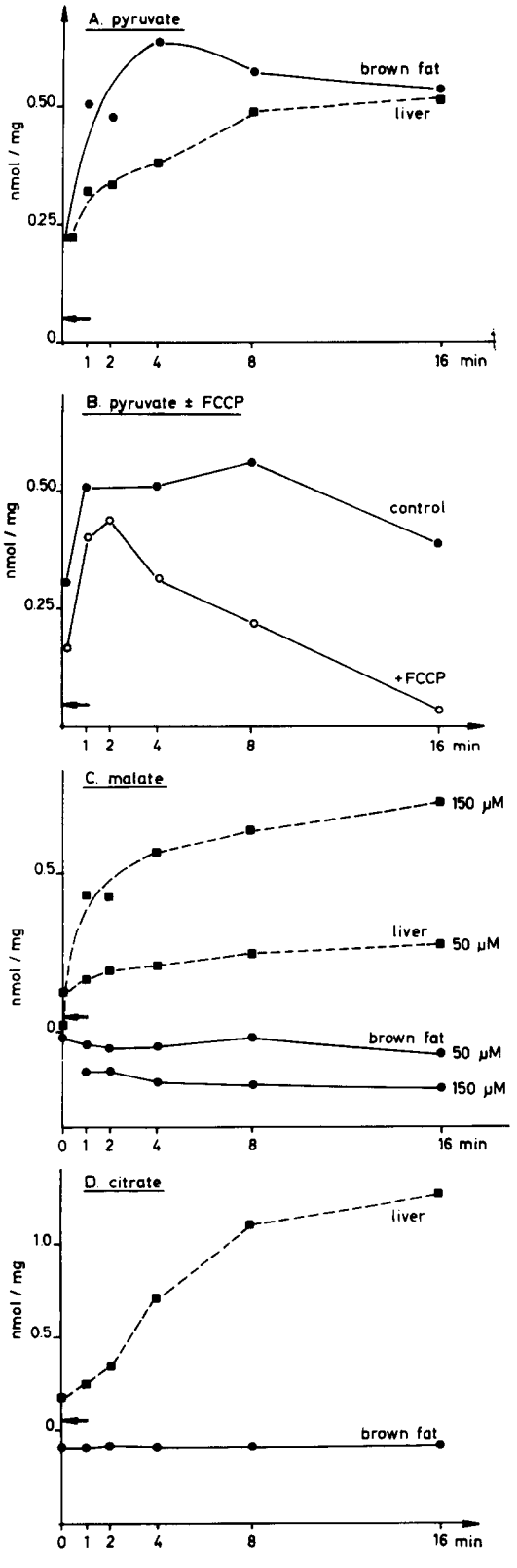
Results and Discussion

Brown adipose tissue from hamsters has previously been shown to have a high capacity for the carboxylation of pyruvate [5], and pyruvate is also a good substrate for isolated hamster brown adipocytes [14]. Intermediates of the citric acid cycle are, however, very poorly oxidised in hamster brown fat mitochondria [8], although the enzymes of the cycle have a high capacity [15]. This would seem to indicate a permeability barrier to entry of the intermediates.

Characterisation of substrate anion uptake

In Fig. 1, results are presented which are indicative of there being a relatively extensive permeability to phosphate [cf. 16] and pyruvate in hamster brown fat mitochondria. Indeed, the rate of swelling in pyruvate was considerably in excess of that in rat liver mitochondria, where it has been extensively studied (for a review, see Ref. 17). In contrast, a very limited permeability to citric acid cycle intermediates was observed such that the rates of swelling with these intermediates were close to zero. This is clearly not due to a limitation in uptake of the transport counter-ion phosphate, but rather to the substrate anions themselves. Parallel experiments under identical conditions with rat liver mitochondria demonstrated that the citric acid cycle intermediates were well transported, as expected (Fig. 1).

Since it is difficult to quantitate the extent of substrate uptake from passive permeability studies of changes in absorbance, respiration-driven uptake of substrate anions was carried out at low concentrations of the relevant anions. Fig. 2A



shows a comparison of ^{14}C-pyruvate uptake in hamster brown fat and rat liver mitochondria. The uptake reached a steady-state level of about 0.5 nmol/mg after 2–4 min and was shown, as expected, to be dependent upon a respiration-linked pH gradient, such that FCCP prevented accumulation of pyruvate. A rapid initial uptake and immediate release was seen when FCCP was added together with the pyruvate (Fig. 2B).

In Figs. 2C and 2D, similar experiments are shown for ^{14}C-malate and ^{14}C-citrate. In these cases, rat liver mitochondria (as earlier demonstrated [17]) accumulated the anions rapidly to a steady-state level, but no respiration-driven accumulation of malate or citrate could be measured in parallel incubations the brown fat mitochondria. This could not be due to competition with the respiratory substrate, since, in the case of brown fat, this was glycerol-3-phosphate, which is oxidised outside the matrix, nor could it be due to inhibition of the carriers by, e.g., acyl-CoAs [18], since albumin was present in the medium. The completely negative result suggests that the slight uptake observed in the swelling experiments was perhaps a slow diffusion process.

It thus appears that hamster brown fat mitochondria, in contrast to rat liver mitochondria, are not designed to oxidise cytosolic citric acid cycle intermediates, but rather to combust acetyl-CoA derived from β-oxidation. The results support our earlier contention [5] that the physiological means of elevating the concentrations of citric acid cycle intermediates in the brown fat mitochondria is through the internal anaplerotic action of pyruvate carboxylase. At the onset of thermogenesis, the mitochondria are then able to accommodate for the rapid influx of acetyl-CoA from fatty acid degradation [4].

Fig. 2. Respiration-driven uptake of radioactively labelled pyruvate (A and B), malate (C) and citrate (D) into hamster brown adipose tissue mitochondria and rat liver mitochondria. For pyruvate, the substrate concentration was 50 μM; for malate, 50 and 150 μM are shown, and for citrate 150 μM is depicted. In (B), the results are for brown fat mitochondria ± 4 μM FCCP added together with the pyruvate. A mitochondrial matrix volume (sucrose-impermeable space) of 1 μl/mg has been assumed [30,31]. Based on this value, the arrows on the ordinate indicate the levels which would be expected from passive diffusion of 50 μM (A, B, C) or 150 μM (D) of the anion. Phosphate and malate were present as required (see Methods and legend to Fig. 1).

Temperature dependence of uptake

Since the hamster is a hibernator whose internal organs must consequently be able to function at temperatures close to zero, we have investigated the temperature dependence of pyruvate uptake. The results are shown in Fig. 3A. The Arrhenius plot obtained was linear, and the apparent activation energy was 71 kJ/mol. The Q_{10} value, calculated according to the formula:

$$Q_{10} = \exp\left(-E_{act} \cdot \frac{300\text{ K} - 310\text{ K}}{300\text{ K} \cdot 310\text{ K}} \cdot \frac{1}{R}\right)$$

for the temperature interval 27–37°C, was 2.5. In comparison, the apparent activation energy for pyruvate carboxylation was 74 kJ/mol (data not shown), i.e., a value not significantly different from that for pyruvate permeation, and in accordance with the possibility that transport could be the rate-limiting step for pyruvate carboxylation. As thus both respiration-driven pyruvate uptake (Fig. 2A) and pyruvate carboxylation proceed at low temperatures, whereas respiration-driven malate uptake is absent (Fig. 2C), it is feasible to think that even at hibernating temperatures, pyruvate carboxylation provides condensing partner for acetyl-CoA in vivo.

In in vitro investigations of fatty-acid oxidation in these, as in other, mitochondria, it is, however, usual and convenient to supply the citric acid cycle by adding malate for the endogenous generation of oxaloacetate [6–9]. We attempted to study the temperature dependence of malate uptake, but as permeation was so slow, the results were difficult to quantitate, although the malate permeation just observable in osmotic swelling experiments at 33°C (cf. Fig. 1) was virtually absent at lower temperatures (not shown). The slow permeation did not appear to be inhibited by butyl-malonate, again supporting a non-carrier mediated process (not shown).

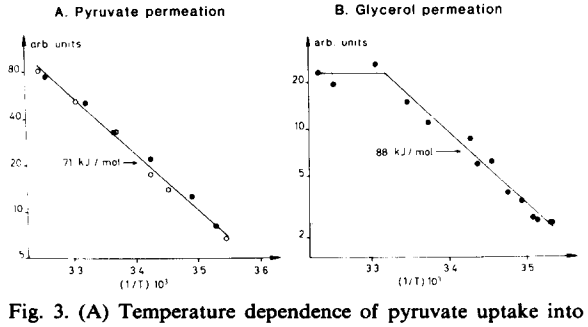


Fig. 3. (A) Temperature dependence of pyruvate uptake into hamster brown fat mitochondria. The results are given as an Arrhenius plot of data obtained from two independent experiments (the different symbols) of osmotic swelling in ammonium pyruvate. (B) Temperature dependence of glycerol permeation into brown fat mitochondria. The results are given as an Arrhenius plot of data obtained from osmotic swelling in 250 mM glycerol, under conditions otherwise identical to those used in Fig. 1.

Since the results above, i.e., the absence of respiration-driven malate uptake, but the presence of a slow malate permeation in isoosmotic swelling experiments, suggested that the malate uptake observed was perhaps a non-carrier-mediated diffusion process, we determined the temperature dependence for permeation of the diffusible molecule glycerol, as a model substance for diffusion processes (Fig. 3B). A strong-temperature dependence (apparent activation energy 88 kJ/mol and a Q_{10} of 3.1) was observed at temperatures below 30°C, demonstrating that this diffusion process is presumably restricted by changes in, e.g., lipid fluidity. We have earlier observed decreased fluidity with decreasing temperature in these mitochondria by the use of spin-labelled fatty acid probes [19,20]. Thus, even a diffusion-mediated uptake may show a high temperature dependence, and so the uptake of the negatively charged malate anion by a slow diffusion process could reveal high temperature sensitivity.

Metabolic consequences of limited malate uptake

It has earlier been shown from our laboratory [7] that the oxidation of palmitoyl-carnitine by hamster brown fat mitochondria can proceed rapidly even in the absence of exogenously added condensing partner (malate). The product is then acetate, which results from the activity of an acetyl-CoA hydrolase. This activity was not found in rat or guinea pig brown fat [7], but was found in lamb [21] and is thus not a hibernation characteristic. At low temperatures, however, acetate was the major product of palmitoyl-carnitine degradation in hamster brown fat mitochondria even in the presence of 3 mM malate [7]. It was therefore suggested that acetyl-CoA hydrolase was present in hamster brown fat in order to enable fatty acid β-oxidation to proceed at low temperature when

the citric acid cycle appeared not to function, perhaps because of "the many diffusion-limited steps occurring in the matrix" [7,22].

In view of the results described above, an alternative explanation for the lack of citric-acid cycle activity in vitro at low temperature presented itself: at the constant malate concentration used at all temperatures in the earlier study, malate permeation became the rate-limiting step at the lower temperatures, and the observed acetate formation was due to lack of oxaloacetate (malate) in the isolated mitochondria.

This hypothesis was investigated based on the following reasoning. When palmitoyl-carnitine is partially degraded to acetate or is completely combusted through the citric acid cycle, the amount of oxygen consumed is, of course, different and this can be used to indicate the extent of palmitoyl-carnitine degradation. An example of this can be seen in Fig. 4, where the extent of respiration (-basal) of a fixed amount of palmitoyl-carnitine at 23°C in the presence of 20 mM malate was about 3 times that seen when malate was absent (as expected from calculations). The rate of oxidation was also considerably faster when malate was present.

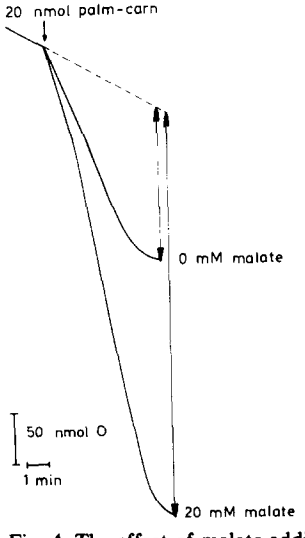


Fig. 4. The effect of malate addition on the extent and rate of palmitoyl-carnitine oxidation in hamster brown fat mitochondria. The temperature was 23°C. For details, see Methods. When compensated for basal respiration, the amount of oxygen consumed in the presence of 20 mM malate was 2.6-times that in the absence of added malate.

Experiments were performed such that hamster brown fat mitochondria were allowed to oxidise a fixed amount of palmitoyl-carnitine in the absence of or in the presence of different concentrations of added malate. The experiments were carried out at five different temperatures between 37 and 8°C. The rates of oxidation and the amounts of oxygen consumed were calculated.

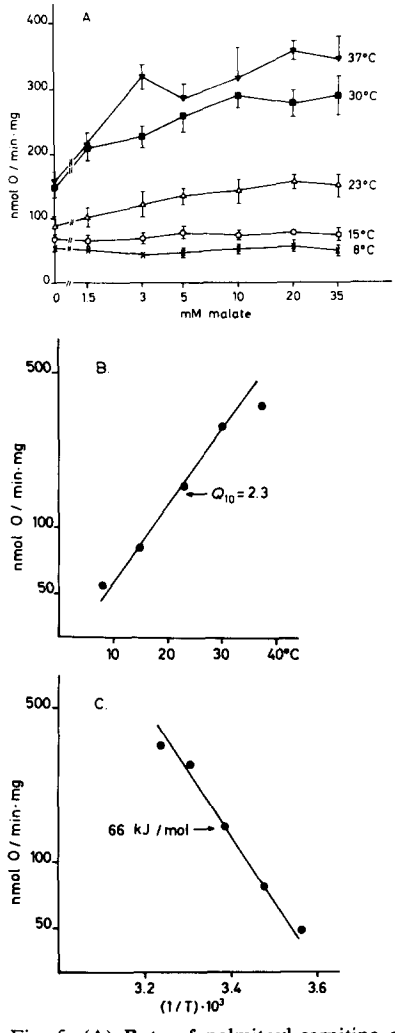


Fig. 5. (A) Rate of palmitoyl-carnitine oxidation in hamster brown fat mitochondria as a function of temperature and malate concentration. The results were obtained from experiments as those depicted in Fig. 4 and are the means ± S.E. of 3–8 experiments at each temperature and concentration. The basal rate of oxidation prior to palmitoyl-carnitine addition was not subtracted, but was negligible in comparison with the measured rates. (B) The maximal rate of respiration as a function of temperature. Note the logarithmic scale. (C) Arrhenius plot of the above data.

In Fig. 5A, the rates of oxidation under the different conditions are shown. The rates were naturally higher at the higher temperatures, but it is notable that only at temperatures above 15°C was there an effect of malate concentration on the oxidation rate. It would thus seem that at low temperatures the rate-limiting step is changed from β-oxidation to a step subsequent to this process, and as the citric-acid cycle is functional at low temperatures (see below and Fig. 6), it may be that the respiratory chain activity is here rate-limiting, whereas at higher temperatures, the mitochondria are able to simultaneously oxidise the additional NADH and $FADH_2$ produced by the citric acid cycle. The Q_{10} value for the influence of temperature on the maximum respiration rate was 2.3 (Fig. 5B). This is in agreement with the Q_{10} value for intact cells [23,24]. An Arrhenius plot of the data (Fig. 5C) gave an apparent activation energy of 66 kJ/mol.

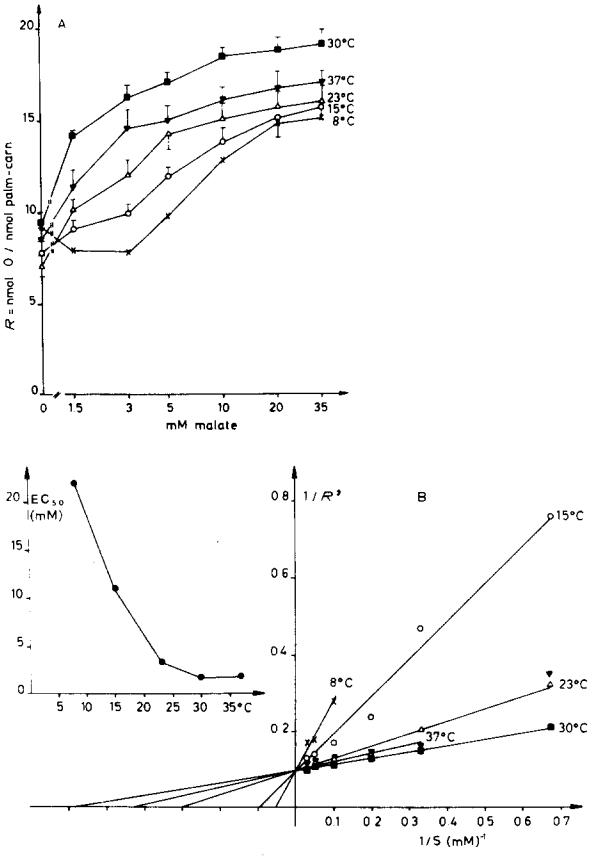


Fig. 6. (A) Extent of palmitoyl-carnitine oxidation as a function of temperature and malate concentration in hamster brown fat mitochondria. The results are from the same experiments as in Fig. 5. The basal oxygen consumption was subtracted. R is the ratio: nmol O consumed per nmol palmitoyl-carnitine nominally added. (As seen, some hydrolysis of the carnitine ester had occurred, resulting in R-values lower than the theoretical; the relative increase in R with increasing malate is, however, as theoretically expected.) (B) Pseudo-Lineweaver-Burk plot of the above data. For each temperature, R' is defined as R (at given malate concentration) minus R (at 0 malate). (C) The EC_{50} values for malate as a function of temperature. The curve is based on the intersection of the lines in (B) with the abscissa.

In Fig. 6A, the amount of oxygen consumed under the different conditions is depicted. It was evident that at high malate concentrations all curves tended towards the same value, a value which was 2–3 times that in the absence of malate. This thus demonstrates that the citric-acid cycle was indeed capable of functioning at low temperature (provided that an adequate level of malate (i.e., oxaloacetate) was maintained) and that entry into the citric acid cycle clearly had preference over acetate formation in agreement with the affinities and capacities of citrate synthase and acetyl-CoA hydrolase in these mitochondria [24].

This is even more apparent when the results from Fig. 6A are linearised in the form of a pseudo-Lineweaver-Burk plot (Fig. 6B). There is then no doubt that the maximum amount of oxygen combusted (per mol palmitoyl-carnitine) at all temperatures at infinite malate concentration would be the same. From Fig. 6A, it was clear that as the temperature was decreased, the EC_{50} values for malate were markedly increased. From Fig. 6B, the EC_{50} values for malate for the different temperatures were read off from the figure and the change in the EC_{50} values with temperature is shown in Fig. 6C. It may be noted that for a 10° decrease in temperature, 3.2-times more malate is needed for equal effects. It may not be a coincidence that the Q_{10} value for glycerol permeation was similarly high (3.1).

The results demonstrate that hamster brown fat mitochondria are indeed capable of completely combusting fatty acids even at the low temperatures found in the animal during the early stages of arousal from hibernation, and that the restriction on complete combustion, which led to acetate production [7], was a metabolic consequence of the unusually low permeability to malate of these

mitochondria, which leads to a need for excessively high concentrations of malate at low temperatures in in vitro studies.

Conclusions

We have here shown that the di- and tricarboxylate anion permeases of hamster brown fat mitochondria are poorly developed. This has several metabolic consequences.

In recent years, brown adipose tissue (from rats and mice) has been shown to have an extremely high capacity for the de novo synthesis of fatty acids [25–27]. However, the brown adipose tissue from hamsters was shown to have a considerably lower capacity, and this observation was unexplained [28]. In most tissues, the requisite cytosolic acetyl units and NADPH are produced from citrate which has been transported out from the mitochondria. (Acetyl-carnitine could perhaps function as an alternative acetyl source, but an additional source of cytosolic NADPH would then be required.) Thus, our observation of exceedingly low mitochondrial permeases for malate and citrate is clearly readily compatible with the reported observations, and may indeed provide at least a partial explanation for the inability of hamster brown fat to show high rates of lipogenesis.

Due to the low malate permeability, the adequate provision of condensing partner at low temperatures necessitates extremely high concentrations of malate in vitro, but when this is fulfilled, fatty acid oxidation proceeds to CO_2 and water even at hibernating temperatures. An adequate provision of oxaloacetate in vivo, probably via pyruvate carboxylase, would thus probably preclude acetate formation in the animal even at low body temperatures. Therefore, the role of the enzyme originally described as an acetyl-CoA hydrolase [29] would not seem to be that of an acetate producer during arousal in the hamster. A recent investigation in our laboratory (Alexson, S. and Nedergaard, J., unpublished data) indicates that both short- and medium-chain, NADH-inhibitable acyl-CoA hydrolases exist in brown fat mitochondria. Their physiological role is as yet unclarified, but it would not seem to be related to hibernation or low-temperature metabolism.

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