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Transport of Reduced Nicotinamide–Adenine Dinucleotide into Mitochondria of Rat White Adipose Tissue

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Mitochondria from rat white adipose tissue were prepared, exhibiting good respiratory control and P/O ratios. They would not oxidize NADH unless *NNN'N'*-tetramethyl-*p*-phenylenediamine was added as a carrier of reducing equivalents. These mitochondria were found to oxidize neither L-glycerol 3-phosphate nor L-glutamate plus L-malate at significant rates. The activity of aspartate aminotransferase in these mitochondria was found to be low compared with that found in rat liver mitochondria. As a consequence of this, the adipose-tissue mitochondria exhibited very low rates of cytoplasmic NADH oxidation in a reconstituted Borst (1962) cycle compared with liver mitochondria.

Lehninger (1951) first showed that mitochondria possessed a permeability barrier to NADH unless subjected to treatment with hypo-osmotic solutions. Purvis & Lowenstein (1961) showed that ^{14}C-labelled NAD^+ and NADH exchange only very slowly with mitochondrial nicotinamide–adenine nucleotides, indicating a low degree of permeability to these compounds. Hohorst and his colleagues suggested that a realistic value for the redox state of the cytoplasm could be obtained by calculation from the [lactate]/[pyruvate] ratio, which gave a value of about 1/2000 for the [NADH]/[NAD^+] ratio (Hohorst, Kreutz & Bücher, 1959; Hohorst, Kreutz & Reim, 1961). Borst (1962) and Klingenberg & von Haffen (1963) used the [acetoacetate]/[β-hydroxybutyrate] ratio to calculate the [NADH]/[NAD^+] ratio for the mitochondrial compartment and estimated it to be about 0.1, i.e. 200 times that found for the cytoplasm. More recent results with rat liver (Williamson, Lund & Krebs, 1967) have shown that the redox states of cytoplasmic and mitochondrial compartments in liver change with starvation and diabetes.

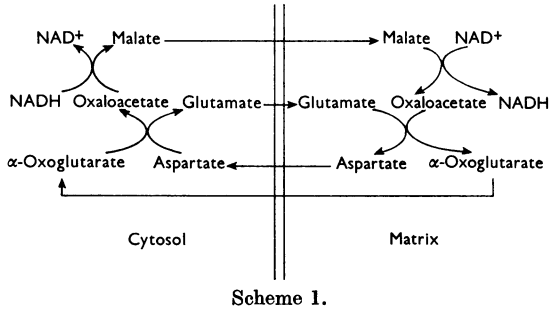
The permeability characteristics of white-adipose-tissue mitochondria have not been investigated. Flatt & Ball (1964, 1966) have demonstrated that when fatty acid synthesis is markedly augmented (adipose tissue incubated in the presence of insulin and glucose) there is a net accumulation of NADH and that this is in the cytoplasmic compartment. Several investigators have suggested that the cytoplasmic [NADH]/[NAD^+] ratio could be very important in the regulation of the direction of flow of glucose carbon atoms.

The present studies were undertaken to evaluate suggested pathways for entrance of reducing power from NADH into the mitochondria. The results indicate that NADH cannot cross the mitochondrial membrane of white adipose tissue. The L-glycerol 3-phosphate–dihydroxyacetone phosphate shuttle does not seem to operate to any significant degree in white adipose tissue, nor does the Borst (1962) shuttle (i.e. a malate–oxaloacetate cycle modified to include a double transamination; see Scheme 1).

MATERIALS

Rats. Epididymal fat pads and perirenal fat were obtained from male Wistar rats weighing 150–250g, fed on a Purina Lab Chow diet. In all experiments the animals were allowed free access to the food before the time of killing, about 10 a.m. In the starvation–re-feeding experiments the animals were fed on a high-carbohydrate diet for 48h after a 72h period of starvation.

Chemicals. Enzymes, coenzymes, glycolytic intermediates and adenine nucleotides were obtained from



Scheme 1.

Boehringer Corp., New York, N.Y., U.S.A., Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. TMPD* was obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

METHODS

Preparation of mitochondria. Isolated cells were prepared from white adipose tissue (epididymal fat pads and perirenal fat) by the method of Rodbell (1964) and suspended in a medium at pH 7.4 containing 0.5M-sucrose, 5mM-tris–HCl, 1mM-EGTA and 2.5% (w/v) bovine serum albumin. The cells were broken by homogenization in an all-glass homogenizer. The mitochondria were isolated from the homogenate by the method of Schneider & Hogeboom (1950). Mitochondrial protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Concentrations of agents used in all the studies appear in the tables or text.

An Aminco–Chance double-beam spectrophotometer (340–373nm) was used for the experiments described in Fig. 6.

Assay of aspartate aminotransferase (*EC* 2.6.1.1). Mitochondria were prepared as described above and washed three times in 0.5M-sucrose containing 1mM-EGTA and 5mM-tris–HCl, pH 7.4. They were then frozen and thawed six times and assayed as described by Bergmeyer & Bernt (1963) with L-malate dehydrogenase. Respiration was measured polarographically with a Yellow Springs model YS 5331 Clark oxygen electrode.

*Abbreviations: TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; EGTA, ethanedioxybis(ethylamino)-tetra-acetic acid.

RESULTS

When oxygen consumption was measured polarographically, white-adipose-tissue mitochondria showed respiratory-control ratios of 6 with pyruvate plus L-malate as substrate and 4 with succinate as substrate (Fig. 1). P/O ratios were measured as 3.1 with pyruvate plus L-malate and 1.89 with succinate. When NADH was added to white-adipose-tissue mitochondria from normally fed or starved–re-fed

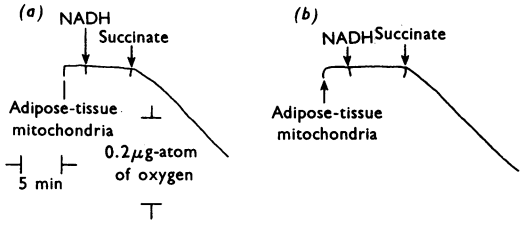


Fig. 2. Inability of white-adipose-tissue mitochondria to oxidize added NADH. Mitochondria (0.9mg of protein) from white adipocytes were suspended in 2ml of the same basal medium as in Fig. 1. To this 5mM-ADP was added, but no substrates were present initially. Additions were (final concns.) NADH, 0.07mM, and succinate, 2.5mM, in each experiment. (*a*) Mitochondria from normally fed rats; (*b*) mitochondria from starved–re-fed rats.

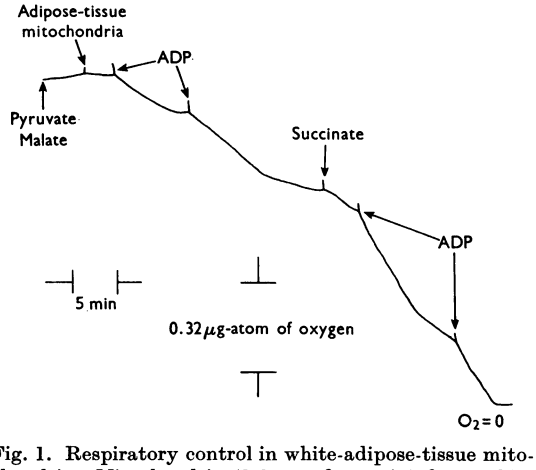


Fig. 1. Respiratory control in white-adipose-tissue mitochondria. Mitochondria (1.0mg of protein) from white adipocytes were suspended in 3.0ml of a medium (pH 7.4, 25°C) containing 125mM-KCl, 20mM-tris–HCl, 2mM-P_i and 1mM-$MgCl_2$, with 2mM-pyruvate and 0.8mM-L-malate added as substrates. Additions were (final concentrations after each addition): ADP, 0.05mM; ADP, 0.1mM; succinate, 1.3mM; ADP, 0.1mM; ADP, 0.2mM. Respiration was monitored polarographically.

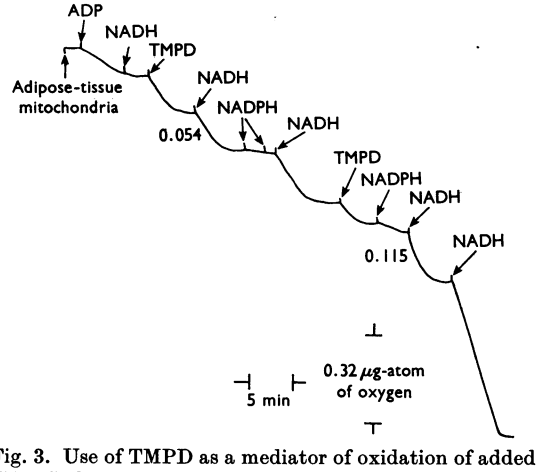


Fig. 3. Use of TMPD as a mediator of oxidation of added NADH by white-adipose-tissue mitochondria. Mitochondria (0.9mg of protein) from white adipocytes were suspended in 3ml of the basal medium as in Fig. 1. Additions were: ADP, 0.8mM; NADH, 0.05mM; TMPD, 0.05μM; NADH, 0.085mM; NADPH, 0.085mM; NADPH, 0.085mM; NADH, 0.085mM; TMPD, 0.05μM; NADPH, 0.17mM; NADH, 0.085mM; NADH, 0.3mM. The numbers adjacent to the trace represent uptake rates expressed as μg-atoms of O/mg of mitochondrial protein.

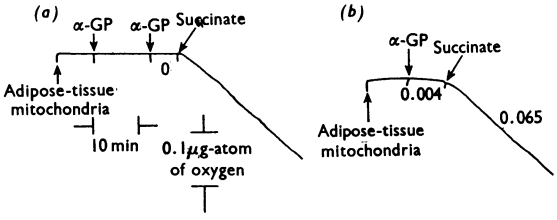


Fig. 4. L-Glycerol 3-phosphate oxidation by white-adipose-tissue mitochondria. Mitochondria (1.0mg of protein) from white adipocytes were suspended in 2ml of the basal medium as in Fig. 1, containing also 0.5mM-ADP. The temperature was 25°C. Additions were as follows. (*a*) L-Glycerol 3-phosphate (α-GP), 2.5mM; L-glycerol 3-phosphate, 2.5mM; succinate, 2.5mM. (*b*): L-glycerol 3-phosphate, 2.5mM; succinate, 7.5mM. (*a*), Mitochondria from normally fed rats; (*b*), mitochondria from starved–re-fed animals. The numbers adjacent to the trace represent uptake rates expressed as μg-atoms of O/mg of mitochondrial protein.

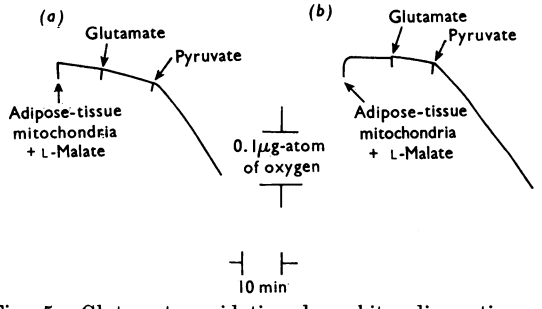


Fig. 5. Glutamate oxidation by white-adipose-tissue mitochondria. Conditions were as described in Fig. 4 except that 2.5mM-L-malate was included in the suspension medium. Additions were as follows. (*a*) (Normal fed rats): L-glutamate, 2.5mM; pyruvate, 4mM. (*b*) (Starved–re-fed rats): L-glutamate, 2.5mM; pyruvate, 4mM.

animals, no significant increase in oxygen consumption was observed (Fig. 2), whereas added succinate was oxidized quite rapidly in both cases.

In Fig. 3, the addition of 50μM-TMPD permitted the mitochondria to oxidize reducing equivalents of NADH but not of NADPH. Addition of TMPD alone results in a small uptake of oxygen, as can be seen when the second addition of TMPD was made in Fig. 3. This is due to the predominance of the reduced form in the added TMPD, which is oxidized by the mitochondria. The double-beam-spectrophotometric experiments (Fig. 6) also confirmed the inability of adipose-tissue mitochondria to oxidize NADH.

White-adipose-tissue mitochondria from normally fed and starved–re-fed rats have a low capacity for the oxidation of L-glycerol 3-phosphate (Fig. 4). Similarly, these mitochondria showed little respiratory activity with glutamate as substrate even in the presence of L-malate (Fig. 5), whereas added pyruvate was oxidized rapidly. Investigation of the aspartate aminotransferase activity in a broken preparation of rat adipose-tissue mitochondria showed it to be 2–5m-units/mg of mitochondrial protein for normally fed rats and 5m-units/mg of mitochondrial protein for the starved–re-fed animals, as compared with 3.0units/mg of mitochondrial protein from a similar preparation from liver.

When a Borst shuttle system (see Scheme 1) for NADH oxidation was reconstructed by addition of ADP and P_i and the sequential additions of L-malate, malate dehydrogenase, L-glutamate and aspartate aminotransferase to liver mitochondria the rate of oxidation of added NADH on the addition of aspartate was very rapid (Robinson, 1968) and sustained (Fig. 6). White-adipose-tissue mitochondria, however, under the same conditions, gave only a

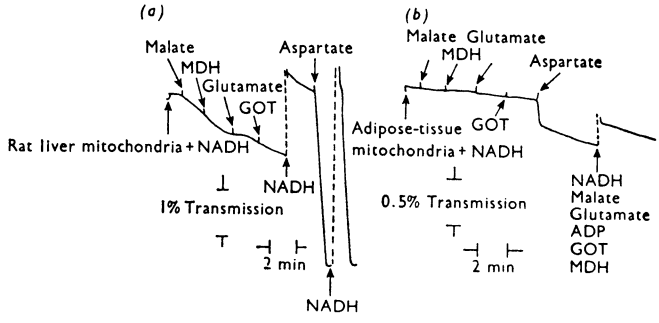


Fig. 6. Comparison of activity of liver and adipocyte mitochondria in a reconstructed Borst cycle system. Rat liver mitochondria (0.1mg of protein) (*a*) and adipocyte mitochondria (1.0mg of protein) (*b*) were suspended in 3.0ml of a medium containing 125mM-KCl, 20mM-tris–HCl and 1mM-potassium phosphate, pH7.4, at 30°C. NADH (0.017mM) and ADP (1mM) were then added (not shown), the reaction being followed by double-beam spectrophotometry (340–373nm). Additions were made as follows: dialysed L-malate dehydrogenase, 2 units (MDH); L-malate, 1mM; dialysed aspartate aminotransferase, 2 units (GOT); L-glutamate, 1mM; L-aspartate, 1mM. Extra NADH additions (0.017mM) were made and the instrument was reset after each addition.

short brisk rate of oxidation of added NADH on addition of aspartate, but this was not sustained and could not be induced by readdition of all the constituents, suggesting the presence of some limitation to the continuation of the cycle. Similar results (not shown) were obtained with white-adipocyte mitochondria from starved–re-fed rats.

DISCUSSION

Flatt & Ball (1964, 1966) have reported that, during the conversion of glucose into fat, reduced coenzymes are produced in excess of those needed to complete not only the synthesis of fatty acid but also of lactate and L-glycerol 3-phosphate. Removal of this excess of reduced coenzymes must occur primarily by reaction with oxygen; therefore a large portion of reduced coenzymes formed at the oxidation of triose phosphate must be reoxidized by oxygen if acetyl-CoA production and fatty acid synthesis are to proceed.

Borst (1963) has reviewed the mechanisms that have been proposed for the oxidation of cytoplasmic NADH by the mitochondria. In most tissues, including adipose tissue (Fig. 2), there is a permeability barrier to NADH (unless the mitochondria are subjected to treatment with hypo-osmotic solutions). Pathways involving substrate cycles are required to translocate the cytoplasmic NADH.

The first type of substrate cycle involving a large change in free energy was shown by Zebe, Delbrück and Bücher (1959) to occur in insect flight muscle. This cycle involves the oxidation of cytoplasmic NADH by dihydroxyacetone phosphate through the cytoplasmic L-glycerol 3-phosphate dehydrogenase reaction. The resulting L-glycerol 3-phosphate then enters the mitochondria and is oxidized by the flavoprotein L-glycerol 3-phosphate dehydrogenase, forming dihydroxyacetone phosphate, which then leaves the mitochondria to re-start the cycle. Since mitochondrial L-glycerol 3-phosphate dehydrogenase has its equilibrium in favour of the oxidized metabolite and the cytoplasmic L-glycerol 3-phosphate dehydrogenase equilibrium favours the reduced metabolite, the cycle is energetically asymmetrical and thus cytoplasmic NADH can still be oxidized even though the cytoplasmic [NADH]/[NAD^+] ratio is much lower than that of the mitochondria. The results in Fig. 4 suggest that this system is not an important pathway in white adipose tissue from normally fed animals, because the mitochondria do not oxidize L-glycerol 3-phosphate at an appreciable rate.

In the starved–re-fed rat there is a small but detectable rate of oxygen consumption when L-glycerol 3-phosphate is the substrate. This could allow a maximal NADH transport rate of 0.8 μmol/min per g wet wt. Lee & Lardy (1965) have demonstrated the presence of a mitochondrial L-glycerol 3-phosphate dehydrogenase in adipocytes from normally fed animals, with an activity similar to that of liver (expressed per mg of mitochondrial protein). Perhaps its activity is significantly higher in the starved–re-fed rat.

The second type of substrate cycle that can be considered is the type where the overall free-energy change is zero. The uneven distribution of intermediates between cytoplasmic and mitochondrial compartments could potentiate the redox asymmetry. The basic requirements are that a symmetrical distribution of the enzymes should exist between the mitochondria and the cytoplasm. Borst (1962) proposed a modified malate-oxaloacetate cycle including a double transamination (Scheme 1). The cycle requires that the mitochondria be permeable to oxoglutarate, malate, glutamate and aspartate and have both cytoplasmic and mitochondrial aspartate aminotransferase and L-malate dehydrogenase (Halperin, Robinson, Martin & Denton, 1969; R. Martin & R. M. Denton, personal communication).

In order to demonstrate this shuttle for the oxidation of cytoplasmic NADH, it was decided that no direct oxidant or pair of oxidants should be added to the system, but rather that the mitochondrial preparation should provide the oxidants as far as possible, as would be the case *in vivo*. The redox changes were followed by double-beam spectrophotometry. As shown in Fig. 6, with adipose-tissue mitochondria, the rate of oxidation was very low when the mitochondria were suspended in the potassium chloride–tris medium in the presence of ADP, P_i and NADH. The rate was not much increased by the addition of L-malate and L-malate dehydrogenase. The addition of aspartate aminotransferase and glutamate provided little further stimulation, although a higher rate was obtained transiently after the addition of aspartate. This initial rate probably corresponds to the transamination of oxoglutarate that was present in the mitochondria, a supply that cannot be sustained. The requirement for added aspartate in the experiment probably reflects the rather high K_m (4.0mM) for aspartate of the aminotransferase (Henson & Cleland, 1964); the K_m for oxoglutarate is considerably lower (0.5mM). The failure of adipose-tissue mitochondria to respire in the presence of L-glutamate and L-malate (Fig. 5) and the low activity of aspartate aminotransferase found in adipose-tissue mitochondria suggests that this enzyme is the limiting component in this cycle.

Flatt & Ball (1964, 1966) calculated that adipose-tissue mitochondria must oxidize NADH at a rate of 0.625 μmol/min per g wet wt. of tissue. The activity of aspartate aminotransferase reported here would sustain a rate of 0.56–1.4 μmol/min per g wet wt., a value close to this experimental observation.

TMPD was tested with white-adipose-tissue mitochondria to determine if it could accelerate the oxi-

dation of cytoplasmic NADH and also to study its effects on the oxidation of cytoplasmic NADPH. The results reported in Fig. 3 demonstrate that in the presence of TMPD reducing power from NADH is very rapidly transported into the mitochondria and oxidized. Strittmatter (1963) has shown that certain dyes, including TMPD, reoxidize the flavoprotein of the liver cytochrome b_5 reductase very rapidly and therefore permit the oxidation of NADH by the cytochrome b_5 reductase, which is situated on the outer mitochondrial membrane (Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967). Lee, Sottocasa & Ernster (1967) have shown that TMPD is also an acceptor for mitochondrial cytochrome *b* and a donor to cytochrome *c*. On the other hand NADPH is oxidized at an extremely low rate, and possibly is not oxidized by the cytochrome b_5 reductase described above. However, this does not rule out the possibility that other cytoplasmic enzymes might catalyse the reduction of TMPD by NADPH in the intact tissue.

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