PYRUVATE METABOLISM IN MITOCHONDRIA FROM RAT AND HUMAN SKELETAL MUSCLE

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CONTENTS

ABBREVIATIONS

A	absorbance
ADP, ATP	adenosine diphosphate, adenosine triphosphate
μCi	microcurie
CoA	coenzyme A
dpm	desintegrations per minute
ϵ	millimolar extinction coefficient
EDTA	ethylene diaminetetraacetate
FCCP	carbonyl cyanide p-trifluoromethoxyphenyl hydrazone
$NAD(P)^+$	oxidized nicotinamide-adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide-adenine dinucleotide (phosphate)
P/O	phosphate/oxygen
RCI	respiratory control index
S.D.	standard deviation
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
Tris	tris (hydroxymethyl) aminomethane
U	unit

alcohol dehydrogenase	Alcohol:NAD oxidoreductase EC 1.1.1.1.
carnitine acetyltransferase	AcetylCoA:carnitine O-acetyltransferase EC 2.3.1.7
carnitine palmityltransferase	PalmitylCoA:carnitine O-palmityltransferase EC 2.3.1.21
citrate synthase	Citrate oxaloacetate-lyase (CoA-acetylating) EC 4.1.3.7
cytochrome oxidase	Ferrocytochrome c:oxygen oxidoreductase EC 1.9.3.1
glutamate-oxaloacetate transaminase	L-Aspartate:2-oxoglutarate aminotransferase EC 2.6.1.1
α-glycerophosphate dehydrogenase	sn-Glycerol-3-phosphate:(acceptor) oxidoreductase EC 1.1.99.5
hexokinase	ATP:D-hexose 6-phosphotransferase EC 2.7.1.1
isocitrate dehydrogenase	threo-D_s-Isocitrate:NAD oxidoreductase (decarboxylating) EC 1.1.1.41
lipoamide dehydrogenase	NADH:lipoamide oxidoreductase EC 1.6.4.3
lipoate acetyltransferase	AcetylCoA:dihydrolipoate S-acetyltransferase EC 2.3.1.12
malate dehydrogenase	L-Malate:NAD oxidoreductase EC 1.1.1.37
pyruvate dehydrogenase	Pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating) EC 1.2.4.1

CHAPTER 1

INTRODUCTION

1.1. GENERAL

Skeletal muscle is the largest tissue-mass of the human body. Surprisingly, it has achieved little attention from biochemists working in the field of intermediary metabolism. As a result, our knowledge concerning the metabolism of mitochondria from skeletal muscle is rather limited when compared to that of mitochondria of other organs such as heart and, especially, liver. The study of the metabolism of skeletal muscle mitochondria is also of importance due to the fact that a disorder in metabolism of skeletal muscle mitochondria may be one of the causes responsible for a muscle disease.

In the last decennia a number of patients has been described that suffered from skeletal muscle diseases which were attended with abnormalities of muscle mitochondria. These abnormalities, found by electron microscopical examination of muscle biopsy specimens, consisted of an increase in the number of mitochondria, an abnormal ultrastructure or both. Because of the occurrence of these mitochondrial abnormalities these muscle diseases are called "mitochondrial myopathies". In some cases, a biochemical defect in the mitochondria could be established.

At present, a number of methods is available to find out whether a patient possibly suffers from a myopathy in which the mitochondria are etiologically involved. Clinical investigations must be suggestive for the occurrence of a myopathy. Clinical chemical investigations performed at rest and during exercise may indicate a disturbance in the energy metabolism of skeletal muscle. Histochemical and electron microscopical investigations of a muscle biopsy specimen give additional information with regard to the existence of a mitochondrial myopathy.

If the results obtained with these investigations point to the existence of a mitochondrial myopathy, biochemical investigations are needed for the establishment and characterization of the disturbance in mitochondrial metabolism. In Chapter 2 a literature survey will be presented in which all the sofar reported biochemical defects are discussed which were found in mitochondria from patients suffering from a mitochondrial myopathy.

1.2. ENERGY METABOLISM OF SKELETAL MUSCLE

Mitochondria are the cell organelles which catalyze the oxidative catabolism of substrates to CO_2 and H_2O coupled to the synthesis of energy. The main substrates for skeletal muscle mitochondria are fatty acids and pyruvate (1). In the resting and submaximally working muscle oxidation of fatty acids and ketone bodies is the main source of energy supply (2,3). During strenuous exercise glycolysis is accelerated which results in an increased lactate production (4). Concomittantly there is an increase in blood lactate concentration, this increase being dependent on the degree of exercise performed and on the extend of training of the subject performing the exercise (5-8). Lactate does not represent the endproduct of skeletal muscle carbohydrate metabolism. Both resting and exercising muscle contribute significantly to the removal of lactate from the bloodstream (9-16), in addition to other organs such as the liver (17), heart (18) and kidney (19). A considerable part of the lactate is oxidized by skeletal muscle to CO_2 (9). Lactate can only be oxidized to CO_2 by the mitochondria after its conversion to pyruvate. So, oxidation of pyruvate by skeletal muscle mitochondria is, in addition to fatty acid oxidation, an important pathway generating energy necessary for normal functioning of skeletal muscle.

1.3. SCOPE OF THIS INVESTIGATION

The investigations described in this thesis were aimed to develop methods to study the metabolism of muscle mitochondria isolated from small human muscle biopsy specimens. With the aid of these methods it may also become possible to detect and characterize defects in mitochondrial metabolism at the molecular level in biopsy specimens from patients suffering from a myopathy which is supposed to be from mitochondrial origin.
Since it was not always possible to obtain normal human skeletal muscle in quantities sufficient for biochemical studies, a lot of the experimental work was carried out on rat skeletal muscle. The procedures developed were subsequently applied to human skeletal muscle.
Our study of mitochondrial metabolism has been limited to the oxidation of pyruvate and citric acid cycle intermediates. The first step in the metabolism of pyruvate is its oxidation to acetylCoA and CO_2 which is catalyzed by the pyruvate dehydrogenase complex. Pyruvate oxidation was studied by measuring

the rate of $^{14}CO_2$ production from[1-^{14}C]pyruvate. Optimal conditions for measuring pyruvate oxidation were established (Chapter 3).
AcetylCoA formed by the action of the pyruvate dehydrogenase complex is further oxidized through the citric acid cycle. Fluxes through various segments of the citric acid cycle have been measured during pyruvate oxidation. The results are reported in Chapter 4.
Pyruvate can be formed in the cytosol of the muscle cell from lactate taken up from the bloodstream or derived from glucose during glycolysis. In both cases pyruvate and NADH are produced in a 1:1 stoichiometry. Since the inner mitochondrial membrane is impermeable to NADH (20), reoxidation of cytosolic NADH must take place through means of transport shuttles for reducing equivalents. The most important shuttles, the malate-aspartate and α-glycerophosphate shuttles, have been reconstituted and the capacity of these reconstituted shuttle systems for the transport of reducing equivalents and their mutual interaction were studied (Chapter 5).
Oxidation of intra-mitochondrially generated NADH is catalyzed by the mitochondrial respiratory chain. A method for the determination of the cytochrome content in mitochondria isolated from fresh and frozen-stored human muscle biopsy specimens is presented in Chapter 6.
In Chapter 7 the results are reported of the effect of different isolation media on the biochemical and morphological characteristics of mitochondria isolated from rat skeletal muscle. Special attention was focussed on the relation between the rate of pyruvate oxidation and the content of cytochrome c present in the mitochondria after isolation.
A discussion concerning the usefulness of muscle homogenates and isolated muscle mitochondria for the detection of a possible metabolic disturbance with the aid of the methods described in the foregoing chapters is presented in Chapter 8.

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CHAPTER 2

MITOCHONDRIAL MYOPATHIES

2.1. INTRODUCTION

The concept of mitochondrial myopathy was introduced by Luft and co-workers (1,2). They described a patient whose main clinical symptoms were muscle weakness and hypermetabolism from non-thyroid origin. The skeletal muscle mitochondria of this patient had ultrastructural abnormalities (giant mitochondria with densily packed cristae) and a biochemical defect (loose-coupling) could be demonstrated. The term mitochondrial myopathy has mainly been used to describe those myopathies in which ultrastructural abnormalities of muscle mitochondria were found, sometimes associated with accumulation of lipid or glycogen in the muscle cell, but without an identified biochemical defect.
A clear definition of mitochondrial myopathy is lacking. Such a definition can be based on the function of skeletal muscle mitochondria: the oxidative catabolism of substrates coupled to the synthesis of energy. Therefore, we consider a mitochondrial myopathy as a condition which is caused by a disturbance in metabolism of skeletal muscle mitochondria Information concerning mitochondrial metabolism can only be obtained by biochemical techniques This definition therefore implies that a mitochondrial myopathy can only be established by biochemical investigations. The morphological appearance of a diseased muscle cell may be suggestive for a disturbance in metabolism but it cannot be used as a criterion to establisch a mitochondrial myopathy Moreover, mitochondrial morphology may alter in a number of neuromuscular diseases in which there is no detectable evidence for a disturbance in mitochondrial metabolism (3).
This chapter is a survey of the literature concerning those cases in which a disturbance in metabolism of skeletal muscle mitochondria has been established. The mitochondrial myopathies are subdivided according to the reported biochemical defect. At first, defects in pyruvate dehydrogenase complex and the citric acid cycle will be discussed. Thereafter, brief attention will be paid to those mitochondrial myopathies which are characterized by a defect in fatty acid oxidation. Finally, disturbances in the respiratory chain will be discussed.

2.2. THE PYRUVATE DEHYDROGENASE COMPLEX

Oxidation of pyruvate is catalyzed by the pyruvate dehydrogenase complex, a multienzyme complex which is composed of three different enzymes: pyruvate dehydrogenase (E_1), lipoate acetyltransferase (E_2) and lipoamide dehydrogenase (E_3).

The activity of the enzyme complex is inhibited by the reaction products NADH and acetylCoA. In addition to product inhibition, the mammalian enzyme complex is subject to regulation by a phosphorylation-dephosphorylation system, which consists of a kinase and a phosphatase These two enzymes catalyze the interconversion of the enzyme complex between the inactive phosphorylated form and the active dephosphorylated form. Both the kinase and the phosphatase require Mg^{2+} ions for their activity, although in different concentrations. The phosphatase requires Ca^{2+} ions in addition (4,5). The activity of the MgATP-requiring kinase is inhibited by ADP and pyruvate (6,7). The portion of the pyruvate dehydrogenase complex which is in the active form is the resultant of the relative activities of the kinase and the phosphatase and hence will be subject to regulation by the mitochondrial energy level which is reflected by the ATP/ADP ratio (8-13). In addition to the ATP/ADP ratio, the interconversion between the active and the inactive form of the pyruvate dehydrogenase complex is also regulated by the $NADH/NAD^+$ ratio and the acetylCoa/CoA ratio (14-17). More detailed information concerning the regulation of the enzyme complex can be found elsewhere (18-21).

Henning et al. (22) showed that 15 % of the pyruvate dehydrogenase complex was in the active form in the gastrocnemius muscle of normal fed rats. This value decreased to 6 % during starvation. Electrical stimulation and mechanical work increased the percentage of the complex in the active form to 60. Studies with perfused rat skeletal muscle showed a decrease in pyruvate dehydrogenase activity during starvation and during streptozotocin induced diabetes (23,24). The activity of the enzyme increased during electrical stimulation of the perfused muscle. The changes in the percentage of the en ie complex which was in the active form paralleled the changes in the rate of pyruvate oxidation, suggesting that the interconversion of the enzyme complex between the active and inactive form is important for the regulation of the rate of pyruvate oxidation in vivo.

2.3. DISTURBANCES IN PYRUVATE DEHYDROGENASE COMPLEX AND CITRIC ACID CYCLE

Only few patients have been described suffering from a defect in the pyruvate dehydrogenase complex in skeletal muscle. Blass and coworkers (25,26) described a 9-year-old boy who suffered from intermittent ataxia and who had elevated blood levels of alanine and pyruvate but a normal level of lactate. Alanine excretion in urine was also elevated. Morphological investigations showed an increase of lipid droplets within the muscle fibers. Studies with white blood cells and fibroblasts of this patient showed that the rate of $^{14}CO_2$ production from [U-^{14}C]glutamate, [1-^{14}C]acetate and [1-^{14}C]palmitate was similar to that found in controls whereas [1-^{14}C]pyruvate oxidation was severely impaired. In further experiments with sonicated fibroblasts of the patient [1-^{14}C]pyruvate oxidation was measured with ferricyanide as electron acceptor. In this manner only pyruvate dehydrogenase activity (E_1) is measured (27). This activity was less than 20 % of that found in controls. It was therefore concluded that this patient suffered from a deficiency of the first enzyme of the pyruvate dehydrogenase complex: pyruvate dehydrogenase (E_1). Evidence for the involvement of skeletal muscle in the deficiency was obtained by the finding that [1-^{14}C]pyruvate oxidation in the patient's muscle homogenate, measured with ferricyanide as electron acceptor, was only 30 % of that found with controls.

Kark et al. (28) investigated pyruvate oxidation in skeletal muscles from a group of 49 patients suffering from a variety of neuromuscular diseases. Pyruvate oxidation was measured in slices of skeletal muscle by determining the rate of $^{14}CO_2$ production from [2-^{14}C]pyruvate and was compared with the rate of [1,4-^{14}C]succinate oxidation. In 15 out of these 49 patients the rate of pyruvate oxidation was lower than that in controls, whereas the oxidation rate of succinate was normal in 12 out of these 15 patients. The patients having a low pyruvate oxidation rate suffered from a spinocerebellar degeneration or a disease of the motor neuron. Metabolism of cultured fibroblasts was studied in only 3 of the patients, suffering from Friedreich's ataxia. Low rates of $^{14}CO_2$ production from [2-^{14}C]pyruvate but normal rates of [1-^{14}C]palmitate and [U-^{14}C]glutamate oxidation were measured. In 3 other patients suffering from Friedreich's ataxia normal rates of pyruvate oxidation were found in skeletal muscle, which indicates that impaired pyruvate oxidation is not an integral feature of Friedreich's ataxia. It was reported in a subsequent paper (29), dealing with 5 patients suffering from Friedreich's ataxia,

that the activities of both the pyruvate dehydrogenase complex as well as the 2-oxoglutarate dehydrogenase complex were decreased in sonicated fibroblasts. This means that not only pyruvate dehydrogenase but also citric acid cycle activity is impaired in these patients. It was suggested that the underlying mechanism responsible for the low rates of $^{14}CO_2$ production from [1-^{14}C] pyruvate and [1-^{14}C]2-oxoglutarate was a modification in lipoamide dehydrogenase (E_3), an enzyme common to both complexes.

Recently, Robinson et al. (30) reported a deficiency of lipoamide dehydrogenase. A male baby was described who suffered from a persistent metabolic acidosis with increased blood levels of lactate, pyruvate, 2-oxoglutarate and branched chain amino acids. Therapeutic trials with thiamine and a high fat diet were unsuccessful. The child died at the age of 7 months. Investigation of skeletal muscle, liver, heart, kidney and brain tissue revealed an activity of the pyruvate dehydrogenase complex which was reduced to 10-20 % of normal activity. Pyruvate dehydrogenase (E_1) activity was normal, but lipoamide dehydrogenase (E_3) activity was deficient. Also, the 2-oxoglutarate dehydrogenase complex was deficient in all tissues tested.

The patients mentioned sofar suffered from a deficiency of one of the enzymes of the pyruvate dehydrogenase complex, in some cases associated with a deficiency of the 2-oxoglutarate dehydrogenase complex. A deficiency in the phosphorylation-dephosphorylation system which regulates pyruvate dehydrogenase activity has also been reported.

Robinson and Sherwood (31) described a patient with a congenital lactic acidosis with high blood levels of lactate, pyruvate and free fatty acids. The patient died at the age of 6 months. An older sister succumbed at the age of 6 weeks with a metabolic acidosis present from birth. Post-mortem studies were carried out on liver, skeletal muscle and brain tissue. A defect in gluconeogenesis could be ruled out. In addition, in all three tissues tested pyruvate dehydrogenase activity was normal and could be inactivated by preincubation with ATP. However, reactivation by Ca^{2+} and Mg^{2+} was minimal in the patient's skeletal muscle and liver tissue; the estimated activity of the pyruvate dehydrogenase phosphatase being lower than 10 %. It was suggested that the deficiency of the pyruvate dehydrogenase phosphatase in liver and muscle and the normal activity of the kinase would maintain the pyruvate dehydrogenase complex in the inactive form in vivo, thus causing the lactic acidosis

In summary, defects in the pyruvate dehydrogenase complex have been reported which are due to a deficiency of the E_1 or E_3 component of the complex or to a

deficiency of the pyruvate dehydrogenase phosphatase. To our knowledge, no deficiencies of the E_2 component or of the pyruvate dehydrogenase kinase have been reported up to now. A deficiency of E_3 results in a deficient activity of the 2-oxoglutarate dehydrogenase complex. Besides this latter deficiency, no other defects in functioning of the citric acid cycle have been reported in human muscle.

2.4. DISTURBANCES IN FATTY ACID OXIDATION

The myopathies in which a defect in fatty acid oxidation could be established were due either to a deficiency of carnitine (32-38) or to a deficiency of carnitine palmityltransferase.
Carnitine deficiency results in "vacuolization" of muscle fibres due to accumulation of lipid. The carnitine content of skeletal muscle is lowered, whereas blood levels of carnitine are decreased or normal (39). In some cases the carnitine deficiency has also been found in heart (37) and liver (38).
Carnitine palmityltransferase deficiency is associated with no or only a moderate accumulation of lipids in skeletal muscle fibres. In all the cases of carnitine palmityltransferase deficiency reported sofar recurrent episodes of myoglobinuria were observed (40-45). These latter are not noticed in carnitine deficiency.

2 5. THE RESPIRATORY CHAIN

Reducing equivalents generated by the pyruvate dehydrogenase complex, by fatty acid oxidation and by the reactions of the citric acid cycle are oxidized by a multienzyme system often referred to as the respiratory chain. The reactions of the respiratory chain take place in the mitochondrial inner membrane. The pathway of electron transport is schematically outlined in Fig 2.1. NADH is oxidized by a flavoprotein which subsequently transfers its electrons to coenzyme Q. In addition to flavoproteins, non-heme iron proteins are involved in electron transport in this region of the respiratory chain. In the span between coenzyme Q and oxygen electron transport is catalyzed by a class of heme-containing proteins called cytochromes Electrons derived from the flavoprotein mediated oxidation of succinate, α-glycerophosphate and acylCoA enter the respiratory chain at the level of coenzyme Q.
Transport of electrons is coupled to the synthesis of energy, which can be

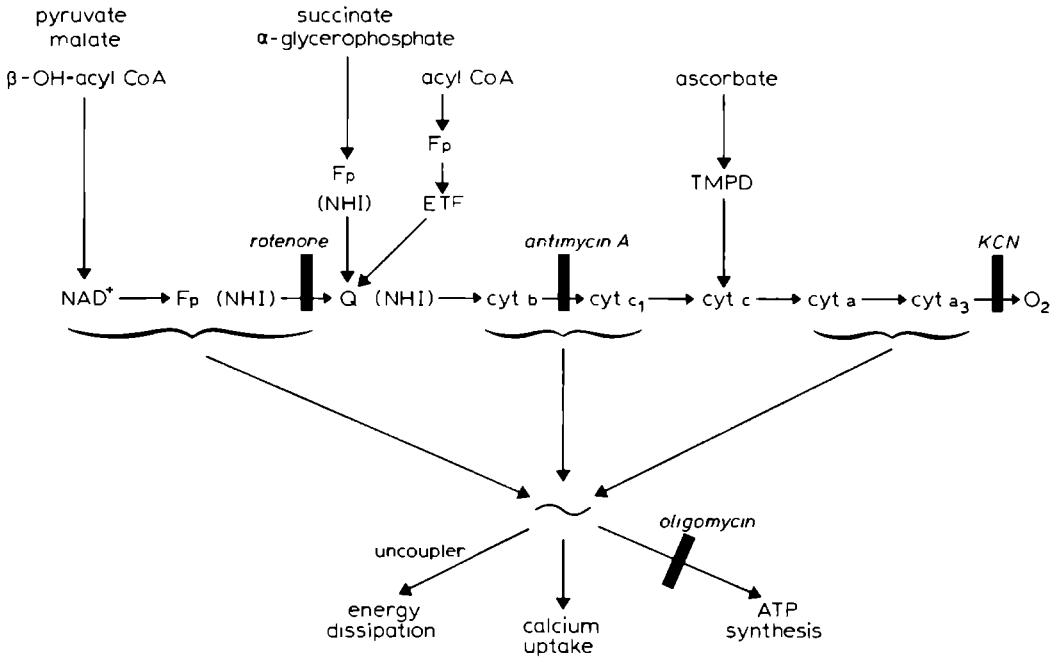

Fig. 2.1. Scheme of the respiratory chain, including the site of action of some inhibitors. Fp: flavoprotein, NHI: non-heme iron, ETF: electron transfering flavoprotein, Q: coenzyme Q, cyt.: cytochrome, TMPD: N,N,N',N'-tetramethyl-p-phenylenediamine.

used for ATP synthesis or calcium uptake. Oxidation of one molecule of intramitochondrial NADH yields three molecules of ATP which are thought to be synthesized at the three phosphorylation sites shown in Fig. 2.1.
Oxidation of the flavoproteins which are reduced during succinate, α-glycerophosphate and acylCoA oxidation yields two molecules of ATP. The efficiency of the phosphorylation process is expressed as the P/O ratio, which is the ratio between the number of ATP molecules formed and the number of oxygen atoms consumed. It will thus be clear that oxidation of pyruvate which yields NADH results in a P/O ratio of three, whereas succinate oxidation leads to a P/O ratio of two.
The oxidation of substrates is coupled to the synthesis of ATP in such a manner that the rate of respiration is controlled by the availability of ADP. This mechanism is called respiratory control. The coupling between oxidation

and phosphorylation is quantified by the respiratory control index, abbreviated RCI. The RCI is defined as the ratio between the rate of oxygen consumption by mitochondria incubated in state 3 and in state 4 conditions. State 3 refers to the situation where mitochondria are incubated in the presence of substrate, phosphate and ADP whereas state 4 represents the situation after the ADP has been phosphorylated to ATP (46).
Proper mitochondrial functioning is dependent both on the presence of all those constituents catalyzing electron transport and ATP synthesis as well as on the regulatory mechanism which controls the coupling between oxidation and phosphorylation. Therefore, the following survey of mitochondrial myopathies which are accompanied by a defect in the respiratory chain has been separated into myopathies characterized by a deficiency of one of the components of the respiratory chain and myopathies characterized by a defect in regulation of oxidative phosphorylation.

2.6. DEFICIENCIES IN THE RESPIRATORY CHAIN

Goldfischer et al (47) described a patient suffering from the cerebro-hepato-renal syndrome of Zellweger. Studies with isolated brain mitochondria of this patient indicated an impaired rate of oxidation of both NADH and flavoprotein linked substrates. Oxygen consumption with ascorbate plus TMPD was comparable to that observed with brain mitochondria from a control subject. With the latter substrate, electrons enter the respiratory chain at the level of cytochrome c and hence a defect in the respiratory chain proximal to cytochrome c was suggested. Since the Zellweger syndrome is a disease in which also muscular abnormalities are noticed, it may be assumed that the defect is also present in skeletal muscle mitochondria. Evidence for the latter as well as information with regard to the localization of the deficiency was obtained from histochemical investigations. Succinate tetrazolium reductase activity with menadion as intermediate electron acceptor was extremely low in skeletal muscle, brain and liver tissue but staining was comparable with controls when phenazine metosulphate was used instead of menadion. Since menadion is thought to accept electrons from coenzyme Q whereas phenazine metosulphate accepts electrons from the succinate dehydrogenase flavoprotein, the deficiency in the respiratory chain was thought to be at the level of the non-heme iron proteins
Mackler et al. (48) studied pyruvate and succinate oxidation in mitochondria

isolated from skeletal muscle biopsy specimens from patients suffering from achondroplasia and in those from healthy controls. Succinate oxidation was comparable, but pyruvate oxidation proceeded with a P/O ratio of 2 6 in controls and 2.1 in the patients mitochondria. The authors therefore suggested a defect in ATP formation at the first phosphorylation site.

Spiro et al. (49) described a hereditary disease of the nervous system and skeletal muscle with progressive ataxia, muscle weakness, reflex anomalies and insidious dementia. The patients were a 46 year old man and his 16 year-old son. Laboratory findings repeatedly indicated increased blood levels of creatine kinase. No ultrastructural abnormalities were present in the skeletal muscle mitochondria. Biochemical studies with isolated skeletal muscle mitochondria from the latter patient revealed low RCI and P/O values with glutamate plus malate as substrates The rate of oxygen consumption was 80 % lower in the patients mitochondria as compared with controls, both with glutamate plus malate and succinate as substrates. Furthermore, succinate oxidation was highly insensitive to inhibition by antimycin A.

Difference spectra of mitochondria after reduction with succinate plus antimycin A did not show a cytochrome b peak which, according to the authors, illustrated the insensitivity of the mitochondria towards antimycin A. Dithionite reduction showed a spectrum with a blue shift of the α-band of cytochrome aa_3 from 609 nm to 592 nm and an extraordinary large absorbtion peak at 432 nm which was ascribed to some kind of "b chromophore" or "an abnormal cytochrome oxidase complex". An alternative and less speculative explanation might be that the mitochondrial suspension was contaminated with hemoglobin and/or myoglobin which, after reduction with dithionite, results in similar spectra (Chapter 6) This may also explain the absence of a spectrum after reduction with succinate plus antimycin A, a combination which is not able to reduce hemoglobin and myoglobin Therefore, the conclusion of the authors that these patients suffered from a cytochrome b deficiency is very speculative.

A second patient suffering from a cytochrome b deficiency was reported by Morgan-Hughes et al. (50). The patient was a 38 year-old man suffering from extreme muscle fatiguability and weakness A muscle biopsy specimen revealed large subsarcolemmal accumulation of bizarre looking mitochondria. Mitochondrial respiration was decreased with different substrates Measurement of cytochromes revealed a decreased content of cytochrome b.

A number of patients has been reported that suffered from a deficiency of

cytochrome aa_3 in skeletal muscle. Monnens et al. (51) described a patient suffering from chronic lactic acidemia, growth faillure and nerve deafness Cytochrome oxidase activity was deficient in muscle tissue. Willems et al. (52) described a girl who suffered from Leigh's encephalomyelopathy. This patient had high blood levels of lactate and pyruvate and an increased lactate/pyruvate ratio. A cytochrome oxidase deficiency could be established in skeletal muscle, the levels of cytochrome b and c were normal. Cytochrome oxidase activity was also reduced in heart muscle but was normal in liver. Skeletal muscle mitochondria varied greatly in size. In large mitochondria irregularly arranged cristae and fine granular electron-dense material were observed. No paracrystalline inclusions were present. The observed high levels of lactate and pyruvate and the increased lactate/pyruvate ratio in cerebrospinal fluid suggested a similar defect in brain

Cytochrome oxidase deficiency has also been reported in trichopoliodystrophy (53). In this case, cytochrome oxidase deficiency was present in muscle, brain and liver tissue and was most probably due to copper deficiency (54).

Van Biervliet et al. (55) reported a patient suffering from a hereditary mitochondrial myopathy with lactic acidosis and a DeToni-Fanconi-Debré syndrome. The patient died at the age of thirteen weeks because of respiratory insufficiency. A deficiency of both cytochromes aa_3 and b was established in skeletal muscle Twin sisters of this patient, exhibiting the same clinical symptoms, had died at the age of eleven weeks, without biochemical investigations being performed. So far, this patient is the only one reported to suffer from a deficiency of two cytochromes.

A patient suffering from a defect in the enzyme system catalyzing ATP synthesis from respiratory chain derived energy was reported by Schotland et al. (56). This patient was a 37 year-old woman suffering from non-progressive weakness present since early childhood. Electron microscopical examination of a skeletal muscle biopsy specimen disclosed numerous mitochondria containing crystalline inclusions. Respiratory rate as well as respiratory control were decreased in isolated mitochondria, but P/O ratio was normal. Addition of uncoupler restored oxidation rates to normal values. Since calcium accumulation kinetics were normal, indicating that energy could be generated in normal amounts by the respiratory chain, a defective ATPase activity was suspected and confirmed by comparing the latter with ATPase activity found in normal skeletal muscle mitochondria.

In summary, a number of deficiencies in the respiratory chain have been

reported. Defects in the region proximal to the cytochromes are rather obscure, which may be caused by the absence of simple analytical procedures for measuring these components of the respiratory chain. Deficiencies of cytochrome aa_3 and cytochrome b as well as a deficiency of ATPase have been established.

2.7. LOOSE-COUPLING

Luft and coworkers (1,2) described a 35 year-old woman suffering from severe hypermetabolism (basal metabolic rate approximately + 200 %) of non-thyroid origin and muscular wasting and weakness. The symptoms of hypermetabolism had been present since childhood. Biochemical studies with isolated skeletal muscle mitochondria from this patient revealed a number of abnormalities, the most important one being that the rate of respiration of the mitochondria was not controlled by the availability of ADP. The high rate of respiration measured in the absence of ADP could hardly be inhibited by oligomycin. However, addition of ADP to these mitochondria resulted in synthesis of ATP. So, respiratory control was absent but phosphorylation could be performed. These mitochondria were in a so-called state of loose-coupling.
Tissue fractionation studies revealed that the amount of mitochondrial protein obtained per gram of muscle weight was three to four times greater than that obtained from skeletal muscle biopsy specimens from normal subjects. Morphological investigations showed an increase in the number of subsarcolemmal mitochondria, an extreme variety in mitochondrial size, an increased amount of densily packed mitochondrial cristae and numerous inclusions of various configurations within the mitochondria.
In subsequent studies with the same patient (57) an attempt was made to isolate the giant mitochondria, which could be seen by electronmicroscopy, by centrifugation of the patient's muscle homogenate at low centrifugational force (5000xg). One quarter of the total amount of mitochondrial protein present in the patient's muscle homogenate was isolated, whereas no mitochondrial pellet was obtained in this manner from muscle homogenates of normal subjects. Although the oxygen consumption per mg protein of the giant mitochondria was lower than that observed with the normal-sized mitochondria of the patient, essentially no differences were noticed with regard to respiratory control index and P/O ratio since both mitochondrial fractions exhibited the phenomenon of loose-coupling.

Addition of the 14 000xg supernatant from a homogenate of the patient's muscle to mitochondria isolated from normal skeletal muscle did not influence the RCI of the latter. Therefore, the presence of an endogeneous factor being responsible for loose-coupling was thought to be unlikely.

Another patient, resembling very much the one described by Luft et al. (2), was described by Haydar et al. (58). It was a 19 year-old girl who also suffered from hypermetabolism, but myopathic symptoms were absent except for a very mild muscle weakness. Morphological examinations of skeletal muscle revealed abnormalities comparable to those reported in Luft's patient: an increased number of mitochondria, many with densily packed cristae and some containing paracrystalline inclusions. Isolated skeletal muscle mitochondria showed the phenomenon of loose-coupling and a high basal ATPase activity. Further investigations showed a normal rate of energy-dependent calcium uptake, but the mitochondria were unable to retain accumulated calcium. The authors suggested therefore that recycling of calcium between the mitochondria and the cytosol might be responsible for a continuous stimulation of respiration in vivo (59). However, since uptake of calcium proceeds at the expense of energy which would otherwise be used for ATP synthesis (60), recycling of calcium would be expected to result in uncoupling rather than loose-coupling.

In addition to the two patients reported sofar, a number of patients has been described that also possessed loosely-coupled skeletal muscle mitochondria but that did not suffer from hypermetabolism (61-66). Clinical symptoms exhibited by these patients ranged from weakness of the pelvic musculature causing difficulties in walking (61) to such a muscle weakness that the patient was confined to a wheelchair (65). In one of the patients, described by Worsfold et al. (64), no clinical symptoms of a myopathy were mentioned. An increased blood level of creatine kinase was demonstrated in this patient, who was examined because of a family relationship with two other patients with clinical symptoms resembling facioscapulohumoral dystrophy, raised blood levels of creatine kinase and loosely-coupled skeletal muscle mitochondria.

In summary, patients have been reported whose skeletal muscle mitochondria exhibited loose-coupling of oxidative phosphorylation. The myopathic symptoms in these patients ranged from mild muscle weakness to complete disability. In addition, severe hypermetabolism may be present. The most profound myopathic symptoms are found in patients who do not suffer from hypermetabolism.

2.8. DISCUSSION

As stated in the introduction, the term mitochondrial myopathy was used in this survey to describe disturbances in metabolism of skeletal muscle mitochondria. In a number of cases the expression of the disturbance was not limited to skeletal muscle but other tissues were also involved. These latter cases therefore seem to represent more generalized "mitochondriopathies".
Since mitochondrial abnormalities may also be present in tissues other than skeletal muscle, the clinical symptoms presented by the patients are subject to great variation. Although all patients have symptoms in varying degree suggestive for a myopathy, there are no specific symptoms which indicate that the origin of the myopathy lies within the mitochondrion. The only exception seems to be hypermetabolism from non-thyroid origin, a symptom of which the relation to mitochondrial functioning is obvious.
Two types of investigations may be performed to obtain information about the possible involvement of mitochondria in a myopathy. Measurement of the changes in blood levels of lactate, pyruvate, fatty acids and ketone bodies during exercise seems very usefull to detect disorders in skeletal muscle metabolism (50,67). The combination of histochemical and electron microscopical examination of a muscle biopsy specimen may indicate whether qualitative and/or quantitative changes are present in skeletal muscle mitochondria.
Morphological criteria alone cannot be used to establish a mitochondrial myopathy. The observed abberations in mitochondrial morphology are unspecific and consist mainly of increases in mitochondrial size and the presence of inclusions within the mitochondria. Sometimes, mitochondrial morphology is abnormal without a detectable biochemical defect in mitochondrial function. This has been found for example in a patient suffering from the Kearns-Shy syndrome (68), in which mitochondrial morphology is frequently abnormal (68-71). It has been shown once that cytochrome oxidase activity was not present within the paracrystalline inclusions, suggesting that they do not have a functional role within the mitochondria (72). Paracrystalline inclusions and the formation of giant mitochondria can be induced by ischemia in rat soleus muscle (73), indicating that mitochondrial morphology may be disturbed by extra-mitochondrial factors (3). On the other hand, in the patient reported by DiMauro and DiMauro (40) who suffered from carnitine palmityltransferase deficiency, no ultrastructural abnormalities of skeletal muscle mitochondria were detected.
The biochemical defect present in the mitochondrial myopathies is the absence

or decreased activity of one of the enzymes involved in oxidative catabolism of pyruvate and fatty acids. Two exceptions can be noticed. Firstly, deficiency of carnitine and secondly the mitochondrial myopathies in which the skeletal muscle mitochondria exhibit loose-coupling. In the latter case, all enzymes seem present but coordination of activities necessary for optimal functioning of the process of oxidative phosphorylation is absent. Deficiencies in pyruvate metabolism were reported in the pyruvate dehydrogenase complex and the respiratory chain, which may be regarded as the first and last part of pyruvate metabolism, respectively. Reports concerning deficiencies in the citric acid cycle, which may be regarded as the intermediate part of pyruvate metabolism, are scarce and limited to defects in the 2-oxoglutarate dehydrogenase complex (29,30). This may be related to the absence of adequate analytical procedures for measurement of citric acid cycle activity in skeletal muscle.

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CHAPTER 3

PYRUVATE OXIDATION IN RAT AND HUMAN SKELETAL MUSCLE MITOCHONDRIA

3.1. INTRODUCTION

A vast amount of knowledge has accumulated during the past years regarding pyruvate oxidation. Regulation of the isolated pyruvate dehydrogenase complex as well as regulation of pyruvate oxidation in intact mitochondria have been studied extensively (1-7). The majority of these studies has been performed with liver and heart muscle mitochondria. There have been hardly any studies concerning pyruvate oxidation in skeletal muscle mitochondria. The investigations performed so far are studies about isolation procedures and hence are only concerned with oxygen uptake and respiratory control (8-13).
Pyruvate oxidation in mitochondria isolated from various tissues and species is known to be stimulated by citric acid cycle intermediates and carnitine (4, 14-18). In this chapter the stimulatory effects of citric acid cycle intermediates and carnitine on pyruvate oxidation in skeletal muscle mitochondria were studied in order to obtain information about the rate-limiting step during [1-^{14}C]pyruvate oxidation. The results obtained with rat skeletal muscle mitochondria are compared with those obtained with human skeletal muscle mitochondria. Application of the results for the diagnosis of a defect in human skeletal muscle pyruvate oxidation is discussed.

3.2. MATERIALS AND METHODS

Preparation of skeletal muscle mitochondria. Rat skeletal muscle mitochondria were prepared from the hind leg muscles of male Wistar rats, weighing 150-200 g and fasted overnight. Human skeletal muscle was obtained from patients with no known neuromuscular disease. The muscle specimens were placed in ice-cold isolation medium containing 250 mM sucrose, 2 mM EDTA, 10 mM Tris, 50 U heparin/ml, final pH 7.4 (SETH-medium). All further operations were carried out at 0-4^{o}. Muscle tissue freed from fat and connective tissue, cut into very fine pieces with scissors and suspended in SETH-medium (1g/10ml). Homogenization was performed by hand with a teflon-glass Potter-Elvehjem type homogenizer. The resulting homogenate was centrifuged for 10 min at 600 xg to remove nuclei and cell debris. After repeating this centrifugation, mitochon-

dria were isolated from the resulting supernatant by centrifugation for 10 min at 14 000 xg. The mitochondrial pellet was rinsed with SET-medium (which is SETH-medium from which heparin is omitted) to remove the fluffy layer, resuspended in SET-medium and centrifuged again for 10 min at 14 000 xg. The mitochondria were finally taken up in SET-medium. Oxygen consumption of the mitochondria, measured as described by Max et al. (13), showed respiratory control indices higher than 4 and P/O ratios between 2.8 and 3.0 with pyruvate and malate as substrates, indicating tight coupling between oxidation and phosphorylation.

Incubations and assays. Mitochondria were incubated in small sealed glass vials at 37° in a shaking waterbath. The basic incubation medium contained in a final volume of 500 µl: 30 mM potassium phosphate, 2 mM EDTA, 10 mM Tris, 5 mM $MgCl_2$, 75 mM KCl, 25 mM sucrose, 20 mM glucose, 2 mM ADP and 1-2 U hexokinase. The final pH was 7.4. Concentrations of substrates are given in the legends to Figures and Tables. Incubations were started by addition of 20-30 µg mitochondrial protein. Small plastic tubes containing 200 µl Hyamine were inserted into the vials in order to trap $^{14}CO_2$ produced during oxidation of [1-^{14}C]pyruvate (19). Incubations were terminated, after 10 or 15 min, by addition of 200 µl 3 M $HClO_4$. The vials were left for an additional 30 min in the shaking waterbath to ensure complete trapping of the $^{14}CO_2$ by the Hyamine. Thereafter, the small plastic tubes with the Hyamine were transferred into 20 ml glass counting vials containing 10 ml toluene and 40 mg Omnifluor. Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter. Under all conditions tested $^{14}CO_2$ production from [1-^{14}C]pyruvate was linear with respect to time and the amount of protein present.

Pyruvate was determined enzymatically according to Czok (20).

Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Statistical analyses were performed with the Student's t test.

Chemicals. The sodium salt of [1-^{14}C] pyruvate was purchased from The Radiochemical Centre, Amersham, England and stored freeze-dried under nitrogen in sealed ampoules at -20° to minimize decomposition (22). Further reagents were purchased as follows: L(-) carnitine and hexokinase (freeze-dried, 40 U/mg) from Koch Light, Colnbrook, England; rotenone and ADP from Boehringer, Mannheim, W-Germany; Omnifluor from NEN Chemicals GmbH, Dreieichenhain, W-Germany and the hydroxide of Hyamine 10X from Packard Instrument Co., Chicago, 111. U.S.A. All other chemicals were of the purest grade commercially

available.

3.3. RESULTS

3.3.1. *Stimulation of pyruvate oxidation by citric acid cycle intermediates.*

The effect of increasing concentrations of malate, fumarate and succinate on the rate of pyruvate oxidation by rat skeletal muscle mitochondria is shown in Fig. 3.1.

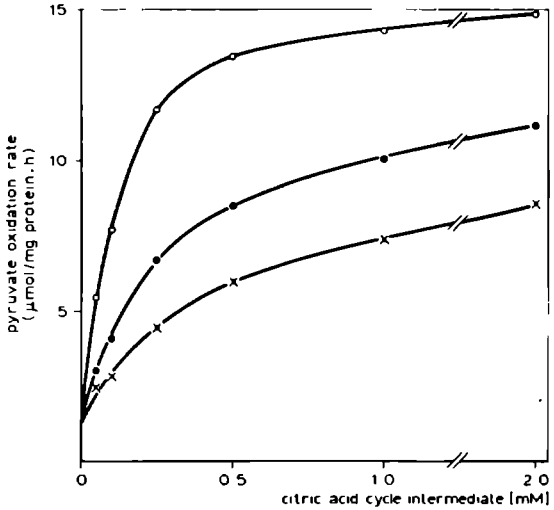

Fig. 3.1. Stimulation of pyruvate oxidation by citric acid cycle intermediates. Rat skeletal muscle mitochondria (54 µg protein/ml) were incubated in the basic incubation medium supplemented with 1 mM pyruvate (0.15 µCi [1-^{14}C]pyruvate). Malate, fumarate and succinate were present in a final concentration as shown in the figure.
o——o malate, x——x fumarate, ●——● succinate.

All the citric acid cycle intermediates tested stimulate pyruvate oxidation although the effect of fumarate and succinate is smaller than that observed with malate. In all instances a final concentration of 1 mM of the added citric acid cycle intermediates is enough to approximate the maximal stimulatory effect which can be obtained with a particular intermediate.
In the presence of malate the rate of pyruvate oxidation measured by the pro-

duction of $^{14}CO_2$ from [1-^{14}C]pyruvate equals the rate of pyruvate disappearance from the incubation medium measured enzymatically, indicating that all the pyruvate taken up by the mitochondria is oxidized to CO_2 (Table 3.1.).

TABLE 3.1.

RATE OF PYRUVATE UPTAKE AND OXIDATION

Rat skeletal muscle mitochondria were incubated in the basic incubation medium supplemented with 1 mM pyruvate (0.15 μCi [1-^{14}C] pyruvate) and 1 mM malate. Pyruvate uptake was measured as the difference in pyruvate concentration in the neutralized deproteinized perchloric acid extracts of the medium before and after incubation. Pyruvate oxidation was measured as described in Materials and Methods.
Values shown are means ± S.D., of the number of experiments given in the parentheses.

Parameter	Rate (μmol/mg protein.h)
Pyruvate uptake	16.3 ± 1.9 (4)
Pyruvate oxidation	15.8 ± 1.2 (6)

3.3.2. *Stimulation of pyruvate oxidation by carnitine.*
The rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate is not only increased by addition of citric acid cycle intermediates but also by addition of carnitine to the mitochondria. AcetylCoA formed by the pyruvate dehydrogenase complex can be transferred to carnitine by the action of carnitine acetyltransferase (23, 24) to form acetylcarnitine and to regenerate mitochondrial CoASH, thereby relieving inhibition of the pyruvate dehydrogenase complex by acetylCoA (25). Carnitine acetyltransferase activity has been shown to be present in muscle mitochondria (26). The effect of carnitine on pyruvate oxidation can be seen in Fig. 3.2. The double reciprocal plot of oxidation rate versus carnitine concentration shows biphasic kinetics. Two apparent K_m values of 0.2 mM and 3.7 mM can be calculated corresponding with a V_{max} of 4.1 μmoles/mg

protein. h and 16.1 μmol/mg protein. h, respectively. The V_{max} reached at infinite carnitine concentration approximates closely the oxidation rate obtained in the presence of 1 mM malate which was 17.0 μmol/mg protein.h in this experiment.

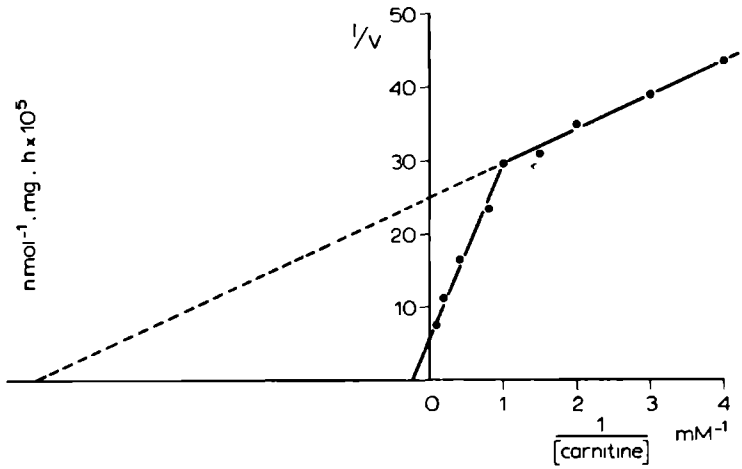

Fig. 3.2. Lineweaver-Burk plot for carnitine stimulation of pyruvate oxidation in rat skeletal muscle mitochondria. 1 mM Pyruvate (0.15 μCi [1-^{14}C]pyruvate) was added to the basic incubation medium.

In the presence of malate no acetate formation was observed. The main products formed in the presence of carnitine were acetylcarnitine and acetate, the latter probably resulting from hydrolysis of acetylcarnitine.

3.3.3. *Pyruvate oxidation without addition of citric acid cycle intermediates or carnitine*

An attempt was made to investigate whether the low rate of pyruvate oxidation measured when no citric acid cycle intermediates or carnitine were added was due to pyruvate oxidation supported by endogenous citric acid cycle intermediates or due to some non-specific decarboxylation of the [1-^{14}C]pyruvate. Therefore, the effect of arsenite, an inhibitor of the pyruvate dehydrogenase complex (27) was studied. Fig. 3.3. shows the effect of arsenite on pyruvate oxidation measured without additions, with carnitine and with malate.

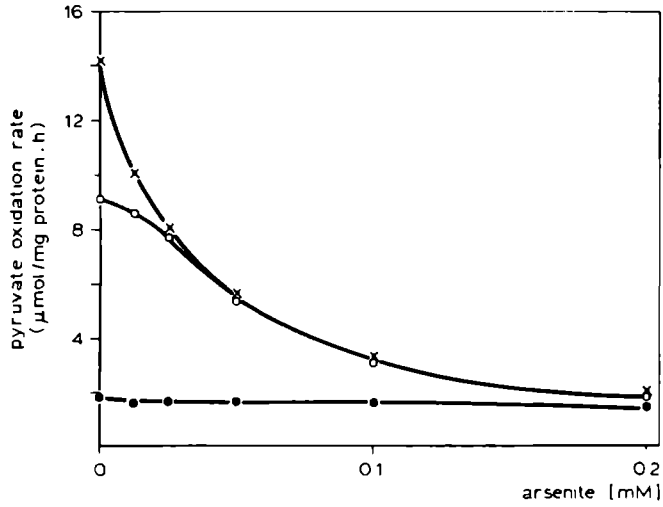

Fig. 3.3. Inhibition of pyruvate oxidation by arsenite. Rat skeletal muscle mitochondria (115 µg protein/ml) were incubated in the basic incubation medium supplemented with 1 mM pyruvate (0.15 µCi [1-^{14}C]pyruvate). Further additions were: ●——● none, o——o 5 mM carnitine, x——x 1 mM malate.

A prompt inhibition of pyruvate oxidation by low concentrations of arsenite is observed in the presence of malate or carnitine. However, hardly any inhibition is observed when pyruvate oxidation is measured without addition of citric acid cycle intermediate or carnitine. Moreover, the residual activity measured under all three conditions is the same. This indicates that part of the pyruvate oxidation measured is insensitive to arsenite inhibition and hence this activity may not represent pyruvate dehydrogenase activity. This is not supported by the effects observed with rotenone or malonate as shown in Table 3.2. The residual activity is completely destroyed by heating of the mitochondria (3 min at 100^{o}) prior to incubation.

TABLE 3.2.

EFFECT OF INHIBITORS ON PYRUVATE OXIDATION WITHOUT ADDITION OF CITRIC ACID CYCLE INTERMEDIATES OR CARNITINE

Rat skeletal muscle mitochondria (46 µg/ml) were incubated in the basic

incubation medium supplemented with 1 mM pyruvate (0.15 μCi [1-^{14}C]pyruvate). Incubations were terminated after 15 min. Inhibitor concentrations were 1 μg/ml rotenone 5mM malonate, o.2 mM arsenite.

Addition	Pyruvate oxidation rate (μmol/mg protein.h)
None	1.5
Rotenone	0.2
Malonate	0.5
Arsenite	1.1

3.3.4. *Pyruvate oxidation in rat and human skeletal muscle mitochondria*

In Table 3.3. the stimulatory effects on pyruvate oxidation of the citric acid cycle intermediates malate, fumarate and succinate are compared with the effect of carnitine in rat and human skeletal muscle mitochondria.

The values for the rate of pyruvate oxidation obtained with human skeletal muscle mitochondria are presented in two manners. Both the absolute values (μmol/mg protein.h) and the relative values (expressed as percentage of those obtained with 1 mM malate) are presented. In the latter manner standard deviations were greatly reduced.

Both in rat and human skeletal muscle mitochondria maximal stimulation of pyruvate oxidation is achieved by malate although this effect is greater in rat than in human skeletal muscle mitochondria. A decrease in stimulatory effect in the order malate › succinate› fumarate is observed both with rat and human muscle mitochondria.

Addition of carnitine results in a stimulation of pyruvate oxidation rate which is in rat skeletal muscle mitochondria half of that observed with malate while in human skeletal muscle mitochondria the same stimulation is achieved as with malate.

In skeletal muscle mitochondria of both species pyruvate oxidation measured in the presence of fumarate or succinate can be further stimulated by addition of carnitine. In the presence of malate no further stimulation by carnitine is observed.

TABLE 3.3.

EFFECT OF CITRIC ACID CYCLE INTERMEDIATES AND CARNITINE ON THE RATE OF PYRUVATE OXIDATION IN RAT AND HUMAN SKELETAL MUSCLE MITOCHONDRIA

Mitochondria were incubated in the basic incubation medium supplemented with 1 mM pyruvate (0.15 μCi [1-^{14}C] pyruvate). Additions of citric acid cycle intermediates (1 mM) or carnitine (5 mM) were as indicated.
Values shown are means ± S.D., of the number of experiments given in the parentheses.
The significance of the difference in oxidation rate in the presence and absence of carnitine was calculated.

Addition	Rat muscle	Human muscle	
	μmol/mg protein.h	μmol/mg protein.h	relative (%)
None	1.1 ± 0.2 (5)	2.0 ± 0.8 (5)	25 ± 3
Malate	15.8 ± 1.2 (6)	7.5 ± 3.5 (6)	100
Fumarate	7.5 ± 1.1 (4)	3.0 ± 1.3 (4)	45 ± 10
Succinate	10.7 ± 0.6 (4)	4.9 ± 2.0 (4)	73 ± 5
Carnitine	8.3* ± 0.8 (5)	7.9* ± 3.8 (6)	105* ± 9
Carnitine + malate	16.4 ± 1.7 (5)	8.8 ± 4.3 (5)	110 ± 7
Carnitine + fumarate	13.0* ± 1.9 (4)	7.1 ± 3.3 (4)	106* ± 12
Carnitine + succinate	14.7* ± 1.4 (4)	7.3 ± 3.8 (4)	107* ± 9

*$p < 0.05$

3.4. DISCUSSION

Pyruvate oxidation in skeletal muscle mitochondria incubated under state 3 conditions without addition of a citric acid cycle intermediate or carnitine is limited by the availability of oxaloacetate. This can be concluded from the stimulatory effect of citric acid cycle intermediates and carnitine. The differences which are observed between the stimulatory effects of malate, fumarate and succinate (Fig. 3.1.) are probably caused by differences in the permeability of the mitochondrial membrane towards these intermediates.
The role of acceptor for acetylCoA generated by the action of the pyruvate dehydrogenase complex is not restricted to oxaloacétate but can also be fullfilled by carnitine (14, 16, 18, 23, 24). The biphasic kinetics of the stimulation of pyruvate oxidation by carnitine shown in Fig. 3.2. points to the action of two different enzymes. The V_{max} reached at infinite carnitine concentration closely approximates the activity found in the presence of malate. So, both malate and carnitine are able to stimulate pyruvate oxidation to the same extent although the effect of malate is reached at far lower concentrations.
Pyruvate oxidation measured without addition of citric acid cycle intermediates or carnitine is supported by endogenous citric acid cycle intermediates. This can be concluded from the observed inhibition by malonate (Table 3.2). Spontaneous decarboxylation cannot be responsible for this residual activity since the latter is inhibited by malonate, rotenone and heating of the mitochondria. The partial insensitivity towards arsenite was attributed in a recent report concerning kidney mitochondria (28) to an "overestimation" of the amount of evolved $^{14}CO_2$. However, since arsenite is a water soluble reagent and the pyruvate dehydrogenase complex is bound to the inner mitochondrial membrane, partial inaccessibility of the pyruvate dehydrogenase complex towards arsenite in intact mitochondria is an alternative explanation.
Citric acid cycle intermediates and carnitine stimulate pyruvate oxidation both in rat and human skeletal muscle mitochondria. Large standard deviations were obtained with human skeletal muscle mitochondria when the activities are expressed on protein base (Table 3.3.). This was reported previously (29). Therefore, the problem of finding a better reference base to express the activities found in human material needs further investigation.
The stimulatory effect of carnitine on pyruvate oxidation in the presence of fumarate or succinate indicates that with these two citric acid cycle inter-

mediates the availability of the acetylCoA acceptor, oxaloacetate, is still rate-limiting for maximal pyruvate dehydrogenase activity. Since no further stimulation of pyruvate oxidation can be achieved by carnitine in the presence of malate, the rate of acetylCoA production is the rate-limiting step during [1-^{14}C]pyruvate oxidation under this condition. The stimulation of pyruvate oxidation by carnitine is equal to that observed with malate in human skeletal muscle mitochondria in contrast to that in rat skeletal muscle mitochondria. This suggests that carnitine acetyltransferase activity is relatively low in rat skeletal muscle mitochondria.

Parallel incubations of human skeletal muscle mitochondria with [1-^{14}C]pyruvate plus malate or carnitine will be the best means of obtaining information about the capacity of human skeletal muscle mitochondria to oxidize pyruvate. It not only permits the diagnosis of an impaired rate of pyruvate oxidation but it also discriminates between a defect in pyruvate dehydrogenase and an impaired capacity of the mitochondria to oxidize acetylCoA via the citric acid cycle. In case of a defect in the respiratory chain it can be expected that pyruvate oxidation will be impaired both with malate and with carnitine added. Then further investigations are indicated to determine whether the defect in pyruvate oxidation is due to a defect in pyruvate dehydrogenase or whether the defect is secundary to a defect in the respiratory chain.

3.5. SUMMARY

Pyruvate oxidation in rat and human skeletal muscle mitochondria was studied by measuring the rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate under state 3 conditions.

The rate of pyruvate oxidation is controlled by the availability of acetylCoA acceptor since addition of citric acid cycle intermediates or carnitine results in a stimulation of pyruvate oxidation.

Pyruvate oxidation proceeds at its maximal rate in the presence of malate since no further stimulation is observed by addition of carnitine. It is concluded that pyruvate dehydrogenase is the rate-limiting step during pyruvate oxidation in the presence of malate.

In human skeletal muscle mitochondria pyruvate oxidation proceeds maximally both in the presence of malate or carnitine. Parallel incubations of these mitochondria with [1-^{14}C]pyruvate plus malate and [1-^{14}C]pyruvate plus carnitine may allow to establish disturbances in pyruvate oxidation and to discri-

minate between defects in the pyruvate dehydrogenase complex and in the activity of the citric acid cycle.

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CHAPTER 4

CITRIC ACID CYCLE ACTIVITY IN RAT AND HUMAN SKELETAL MUSCLE MITOCHONDRIA

4.1. INTRODUCTION

AcetylCoA formed by the pyruvate dehydrogenase complex during pyruvate oxidation is oxidized to CO_2 by the reactions of the citric acid cycle. Although this is a cyclic process, considerable evidence has been obtained that there is no uniform flux through various segments of the cycle. Accumulation of citrate, 2-oxoglutarate and succinate has been reported during pyruvate oxidation in isolated heart (1-4), brain (4-6) and liver (7) mitochondria.
The aim of the experiments described in this chapter was to obtain information concerning citric acid cycle activity in skeletal muscle mitochondria. The method which seemed most suitable for obtaining this information is based on the measurement of $^{14}CO_2$ production from ^{14}C-labeled cycle intermediates. Interpretation of the results obtained requires knowledge about possible accumulation of citric acid cycle intermediates, since $^{14}CO_2$ is only liberated in the isocitrate and 2-oxoglutarate dehydrogenase catalyzed reactions.
Citric acid cycle activity during pyruvate plus malate oxidation was first studied in rat muscle mitochondria. Disappearence of substrates from the incubation medium and accumulation of intermediates were measured by enzymatic procedures. Citric acid cycle activity was also measured after reconstitution of the malate-aspartate shuttle to study the effect of the latter on the accumulation of cycle intermediates. Finally, activities of the various segments of the citric acid cycle were determined by measuring the rate of $^{14}CO_2$ production from ^{14}C-labeled intermediates. With the latter technique citric acid cycle activity was also determined in human skeletal muscle mitochondria.

4.2. MATERIALS AND METHODS

Isolation procedure for skeletal muscle mitochondria, composition of the basic incubation medium and assays of $^{14}CO_2$ production and of protein are described in Chapter 3. All incubations were performed in state 3 conditions. The concentration of phosphate in the medium was 30 mM, which has been shown to result in an optimal rate of malate transport in rat heart mitochondria (8).

Metabolite assays. For the enzymatic determination of metabolites reactions were stopped by addition of 0.2 ml 6 M $HClO_4$ to 1 ml incubation medium. After centrifugation to remove precipitated protein, the samples were neutralized with 4 M KOH. Assays of pyruvate, malate, 2-oxoglutarate and NAD^+ were performed according to standard enzymatic procedures (9) in the neutralized samples. The assay of succinate was based on the oxidation of succinate to fumarate by rat heart mitochondria (10) and coupled to the reduction of cytochrome c. The latter was measured spectrophotometrically at 550 nm. The cuvette contained in a final volume of 1 ml: 10mM Tris-HCl, 5 mM $MgCl_2$, 2 mM KCN, 2 μg rotenone and 90 μM cytochrome c, final pH 7.4. The reaction was started by addition of 20 μl of a suspension of rat heart mitochondria, which were isolated as described for skeletal muscle mitochondria. The mitochondria isolated from one heart were taken up in 1 ml SETH medium. The increase in absorbance at 550 nm was proportional to the amount of succinate. Assays were carried out in the presence of added succinate as standard.

High voltage electrophoresis. High voltage electrophoresis of neutralized samples was performed on strips (40 x 4 cm) of Whatman 3 MM paper for 30 min at 10 mA per strip in 2 M acetic acid adjusted to pH 3.0 with pyridine (11). After drying, the strips were cut in 1 cm pieces which were transferred to glass counting vials. The pieces were shaken vigorously with 5 ml of 0.1 M NaOH for 45 min to extract ^{14}C-labeled intermediates. Thereafter, 10 ml of Instagel were added and radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter.

Chemicals. [1-^{14}C]pyruvate, [2-^{14}C]pyruvate and [U-^{14}C]malate were purchased from the Radiochemical Centre, Amersham, England, [1-^{14}C]2-oxoglutarate from NEN Chemicals GmbH, Dreieichenhain, Germany. Cytochrome c (horse heart), glutamate-oxaloacetate transaminase and malate dehydrogenase were obtained from Boehringer, Mannheim, Germany The latter enzymes were dialyzed against 20 mM potassium phosphate buffer, containing 1 mM EDTA, pH 6.7, to remove ammonium sulphate.

Instagel was purchased from Packhard Instrument Co., Chicago, Ill., U.S.A.

4.3. RESULTS

4.3.1. *Citric acid cycle flux during pyruvate oxidation*

Changes in concentration of citric acid cycle intermediates were measured after various periods of incubation to investigate citric acid cycle flux in

skeletal muscle mitochondria during pyruvate plus malate oxidation. Oxidation of pyruvate is accompanied by a decrease in the concentration of malate, which is due to accumulation of 2-oxoglutarate and succinate (Fig. 4.1).

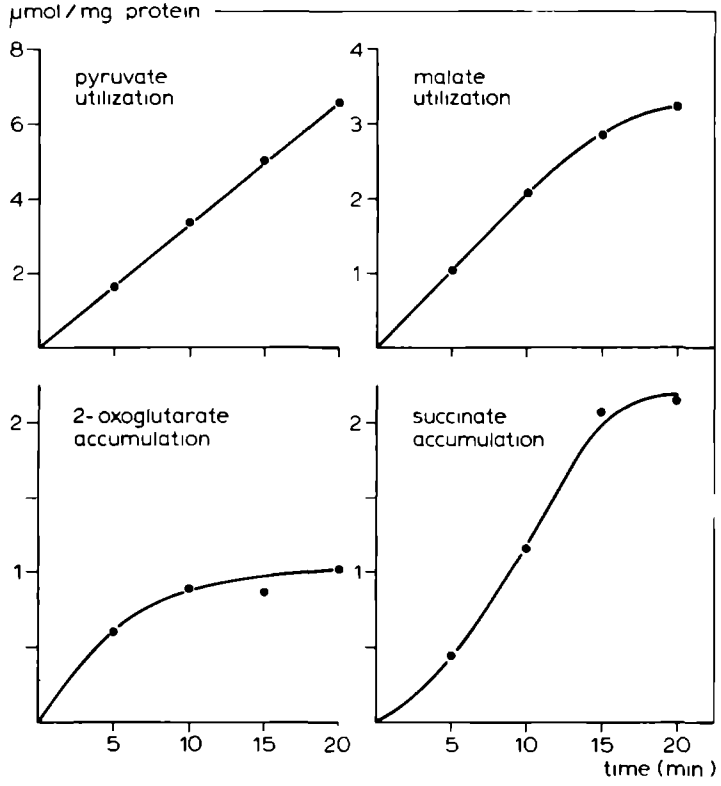

Fig. 4.1. Utilization of pyruvate and malate and accumulation of 2-oxoglutarate and succinate during oxidation of pyruvate plus malate.
Rat skeletal muscle mitochondria (0.13 mg protein) were incubated in the basic incubation medium (final volume 1 ml) supplemented with 1 mM pyruvate and 1 mM malate. Incubations were terminated at the times indicated.

Eventually a steady state may be reached, during which there is no further accumulation of 2-oxoglutarate and succinate nor a further utilization of malate, but the rate of pyruvate oxidation remains constant. The sum of the accumulated 2-oxoglutarate and succinate equals the amount of malate utilized. At any time, citrate, isocitrate and fumarate were not detectable. Fluxes through various segments of the citric acid cycle were calculated from the

changes in concentrations of intermediates (Fig. 4.2).

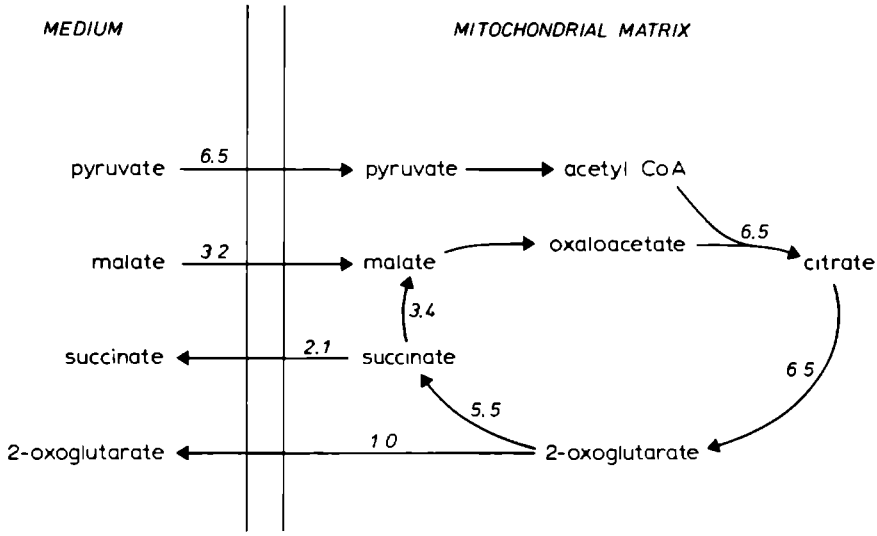

Fig. 4.2. Citric acid cycle flux in rat skeletal muscle mitochondria oxidizing pyruvate plus malate.
Fluxes through the various segments of the citric acid cycle (μmol/mg protein) were calculated from the observed changes in metabolite concentrations between 0 and 20 min of the experiment shown in Fig. 4.1.

Since no detectable accumulation of citrate and isocitrate takes place, the rate of pyruvate oxidation may be assumed to equal the rate of 2-oxoglutarate synthesis. Fluxes through 2-oxoglutarate dehydrogenase and succinate dehydrogenase catalyzed reactions can be calculated from the rate of 2-oxoglutarate synthesis and the accumulation of 2-oxoglutarate and succinate.
Transport of 2-oxoglutarate and succinate across the mitochondrial membrane takes place as a 1:1 exchange with malate (12-14). Since the decrease in malate concentration equals the sum of the concentrations of the accumulated 2-oxoglutarate and succinate, accumulation of these intermediates was assumed to take place in the extra-mitochondrial space, as was shown for rat heart mitochondria (1-3).
The possibility that the 2-oxoglutarate accumulation was due to contamination of pyruvate with parapyruvate (15), a powerfull inhibitor of the 2-oxoglutarate dehydrogenase complex (16), was excluded by the following experiment.

Mitochondria were incubated with 1 mM malate and pyruvate in concentrations ranging from 1 to 10 mM. If pyruvate should be contaminated with parapyruvate, the increase in parapyruvate concentration should result in an increased inhibition of 2-oxoglutarate dehydrogenase. However, no effect of increasing pyruvate concentrations was observed on 2-oxoglutarate accumulation (data not shown).

4.3.2. *Fluxes through the citric acid cycle and the reconstituted malate-aspartate shuttle*

2-Oxoglutarate is an intermediate of two important pathways of skeletal muscle metabolism. It can be oxidized by the 2-oxoglutarate dehydrogenase complex and it can also be used extra-mitochondrially in the reactions of the malate-aspartate shuttle, which is one of the mechanisms for the transfer of reducing equivalents across the mitochondrial membrane (14,17). The observed accumulation of 2-oxoglutarate at the expense of malate (Fig. 4.1) may result from the absence of the cytosolic components of the malate-aspartate shuttle in the incubation medium. To investigate this possibility, an experiment was performed in which the malate-aspartate shuttle was reconstituted by addition of aspartate, NADH, glutamate-oxaloacetate transaminase and malate dehydrogenase to the incubation medium of a mitochondrial suspension oxidizing pyruvate plus malate.

Fluxes through the citric acid cycle and the malate-aspartate shuttle were calculated from the measured changes in concentration of pyruvate, malate, 2-oxoglutarate, glutamate, succinate and NAD^+ after 20 min of incubation (Fig. 4.3).

Reconstitution of the shuttle eliminated 2-oxoglutarate accumulation. Instead, glutamate accumulated. Still considerable accumulation of succinate took place. Flux through the cytosolic glutamate-oxaloacetate transaminase and malate dehydrogenase, calculated from the rate of NAD^+ formation, was twice the flux through the citric acid cycle span from 2-oxoglutarate to oxaloacetate during the 20 min of incubation.

4.3.3. *Measurement of citric acid cycle activity with ^{14}C-labeled substrates*

Information concerning citric acid cycle activity can also be obtained by measuring the rate of $^{14}CO_2$ production from ^{14}C-labeled substrates. The results obtained with this technique in rat and human skeletal muscle mitochondria are presented in Table 4.1.

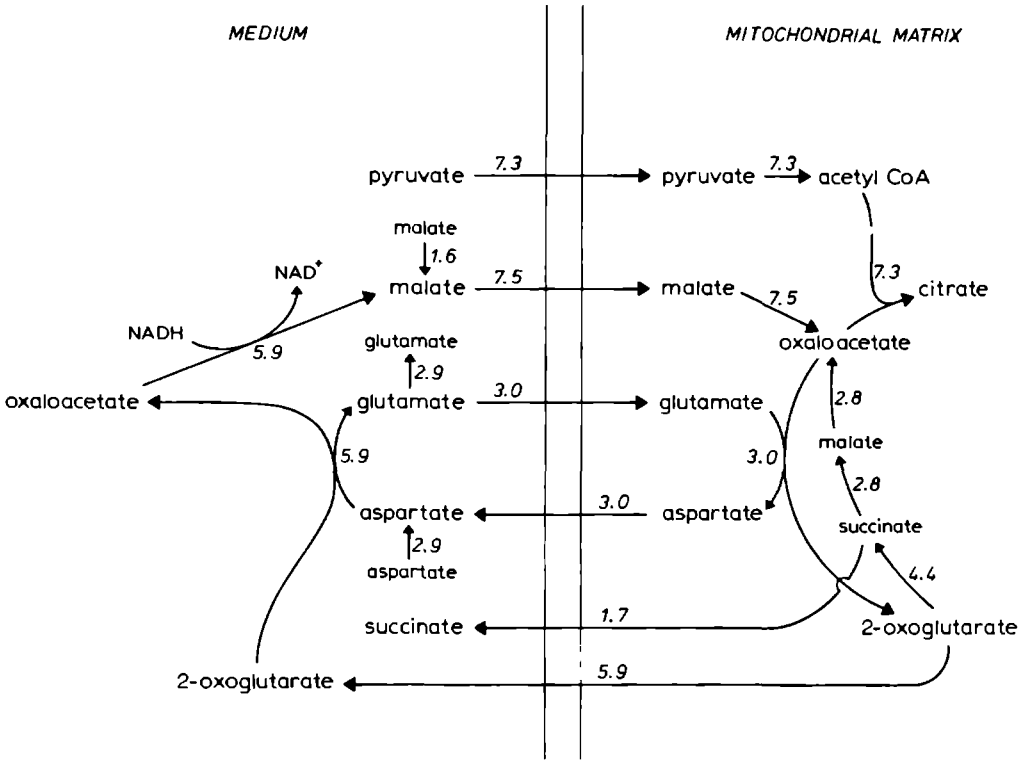

Fig. 4.3. Fluxes through the citric acid cycle and the reconstituted malate-aspartate shuttle.
Rat skeletal muscle mitochondria (0.12 mg protein) were incubated for 20 min in the basic incubation medium (final volume 1 ml) supplemented with 1 mM pyruvate, 1 mM malate, 2mM NADH, 5 mM aspartate, 5 U glutamate-oxaloacetate transaminase and 12 U malate dehydrogenase.
Fluxes (μmol/mg protein) through the citric acid cycle and the malate-aspartate shuttle were calculated from the measured changes in concentration between 0 and 20 min of pyruvate (-881 nmoles/ml), malate (-200 nmoles/ml), 2-oxoglutarate (0 nmoles/ml), succinate (+242 nmoles/ml), glutamate (+349 nmoles/ml) and NAD^+ (+703 nmoles/ml).

$^{14}CO_2$ production was measured after incubation with [1-^{14}C]pyruvate, [U-^{14}C] malate and [1-^{14}C]2-oxoglutarate. With [1-^{14}C]pyruvate as substrate $^{14}CO_2$ is liberated at the pyruvate dehydrogenase complex. $^{14}CO_2$ production from [U-^{14}C]

malate was measured in the presence of malonate to prevent recycling of malate through the cycle. $^{14}CO_2$ is liberated both at isocitrate dehydrogenase as well as at 2-oxoglutarate dehydrogenase. In the presence of [U-^{14}C]malate and arsenite, $^{14}CO_2$ is only liberated at isocitrate dehydrogenase. With [1-^{14}C] 2-oxoglutarate the activity of the 2-oxoglutarate dehydrogenase complex is measured. To compare the citric acid cycle activity with the activity of the pyruvate dehydrogenase complex under identical conditions, malonate was also added to the incubations with [1-^{14}C]pyruvate plus malate (Table 4.1). Both in rat and human muscle mitochondria the rates of pyruvate oxidation are lower in the presence of malonate.

TABLE 4.1.

$^{14}CO_2$ PRODUCTION FROM [1-^{14}C]PYRUVATE, [U-^{14}C]MALATE AND [1-^{14}C]2-OXOGLUTARATE

Assays were performed in the basic incubation medium, final volume 0.5 ml and containing approximately 30 μg mitochondrial protein. Additions were made as follows: pyruvate 1 mM, malate 1 mM, 2-oxoglutarate 1 mM, acetylcarnitine 2 mM, malonate 5 mM and arsenite 2 mM. The assays contained approximately 0.2 μCi of the labeled substrate. Values shown (expressed as μmol $^{14}CO_2$/mg protein.h) are the means $\pm$ S.D. of the number of experiments shown in the parentheses.

Addition	Rat	Human
[1-^{14}C]pyruvate + malate	10.1 $\pm$ 1.2 (6)	6.3 $\pm$ 1.3 (6)
[1-^{14}C]pyruvate + malate + malonate	8.5 $\pm$ 0.9 (6)	4.9 $\pm$ 1.8 (6)
[U-^{14}C]malate + pyruvate + malonate	15.4 $\pm$ 1.4 (6)	6.5 $\pm$ 1.7 (6)
[U-^{14}C]malate + acetylcarnitine + malonate	5.6 $\pm$ 0.8 (6)	6.3 $\pm$ 1.7 (6)
[U-^{14}C]malate + acetylcarnitine + arsenite	3.1 $\pm$ 0.3 (6)	3.8 $\pm$ 0.9 (6)
[1-^{14}C]2-oxoglutarate	14.9 $\pm$ 1.9 (6)	11.1 $\pm$ 3.0 (4)

$^{14}CO_2$ production rates from [U-^{14}C]malate are about equal with pyruvate or acetylcarnitine as acetyl donor in human muscle mitochondria, whereas the rate of $^{14}CO_2$ production from [U-^{14}C]malate with pyruvate is about three fold higher than that with acetylcarnitine in rat muscle mitochondria. The latter suggests that acetylCoA is generated at a three fold higher rate from pyruvate than from acetylcarnitine in rat muscle mitochondria, whereas these rates are equal in human muscle mitochondria.

Both in rat and human muscle mitochondria a divergence exists between the activity of the pyruvate dehydrogenase complex (measured with [1-^{14}C]pyruvate plus malate) and the activity of isocitrate dehydrogenase (measured with [U-^{14}C]malate plus acetylcarnitine and arsenite). In rat muscle mitochondria this may be explained by the different rates at which acetylCoA is generated from pyruvate and acetylcarnitine. The divergence in human muscle mitochondria may be caused by the fact that either less acetylCoA than has been formed enters the citric acid cycle or that citrate accumulates. To investigate this latter possibility, human muscle mitochondria were incubated with malate, malonate and [2-^{14}C]pyruvate. After incubation, metabolites were separated by high voltage electrophoresis. Radioactivity was present in pyruvate, 2-oxoglutarate and succinate, but ^{14}C-citrate was not detectable (Fig. 4.4). [2-^{14}C] pyruvate was used in this experiment since citrate comigrates with malate during high voltage electrophoresis under the used conditions.

4.4. DISCUSSION

4.4.1. *Accumulation of 2-oxoglutarate and succinate during pyruvate plus malate oxidation*

Oxidation of pyruvate plus malate by rat skeletal muscle mitochondria is accompanied by accumulation of 2-oxoglutarate and succinate and utilization of malate. From the changes in concentrations of metabolites (Fig. 4.1) it can be concluded that the citric acid cycle in muscle mitochondria can be divided into three segments: entry of acetylCoA to 2-oxoglutarate synthesis, oxidation of 2-oxoglutarate to succinate and further oxidation of succinate to malate. Moreover, it appears that the fluxes through these various segments are different, especially during the initial stage of incubation. During prolonged incubation the rates of 2-oxoglutarate and succinate accumulation as well as the rate of malate utilization decrease, suggesting that ultimately a steady state will be reached. No detectable accumulation of citrate and isocitrate

took place, which may be related to the absence of a tricarboxylate translocase in muscle mitochondria (18).

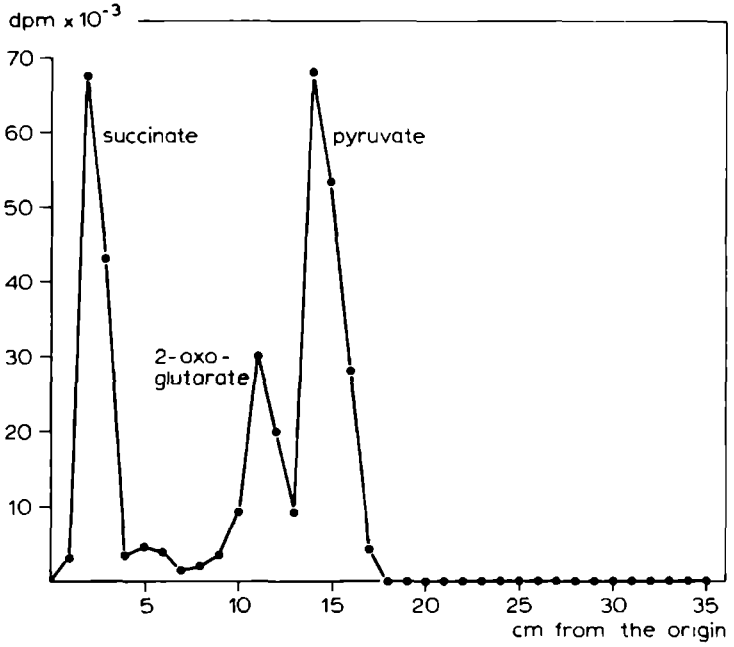

Fig. 4.4. Incorporation of radioactivity from [2-^{14}C]pyruvate in citric acid cycle intermediates.
Human skeletal muscle mitochondria (0.22 mg protein) were incubated for 15 min in the basic incubation medium (final volume 1 ml) supplemented with 1 mM malate, 5 mM malonate and 1 mM pyruvate (0.25 μCi [2-^{14}C]pyruvate). High voltage electrophoresis of a neutralized perchloric acid sample of the incubation medium and subsequent determination of radioactivity were performed as described under Materials and Methods. If present, ^{14}C-citrate should be found at about 5 cm from the origin.

2-Oxoglutarate and succinate are cycle intermediates which can either be oxidized by the reactions of the citric acid cycle or can be transported across the mitochondrial membrane in exchange for malate. The accumulation of these intermediates is, therefore, the resultant of the relative activities of their transport and oxidation pathways. The activity of the 2-oxoglutarate dehydrogenase complex is low during pyruvate oxidation (7), which is probably

due to competition for CoA between the pyruvate and 2-oxoglutarate dehydrogenase complexes. The transport of 2-oxoglutarate and succinate is stimulated by malate (12-14) and will be most active during the initial stage of incubation when the extra-mitochondrial concentrations of 2-oxoglutarate and succinate are low. During incubation the rate of accumulation of these intermediates decreases, whereas the rate of pyruvate oxidation remains constant (Fig. 4.1). Hence, an increasing part of the oxaloacetate necessary for pyruvate oxidation is derived from malate regenerated through citric acid cycle activity during incubation.

Pyruvate and NADH are generated in stoichiometric amounts in the cytosol from lactate taken up from the bloodstream or produced during glycolysis. Therefore, oxidation of pyruvate will be coupled to the oxidation of extra-mitochondrial NADH. One of the mechanisms catalyzing the transport of reducing equivalents across the mitochondrial membrane is the malate-aspartate shuttle (14,17,19). Operation of this shuttle involves the exchange of extra-mitochondrial malate for intra-mitochondrial 2-oxoglutarate. In the presence of the reconstituted malate-aspartate shuttle a considerable part of the oxaloacetate required for pyruvate oxidation is derived from malate formed through the reactions of this shuttle during the 20 min of incubation (Fig. 4.3). The accumulation of 2-oxoglutarate is eliminated but now a rise in glutamate and a decrease in aspartate levels is observed. The changes in concentration of these latter metabolites are in the same direction as observed in heart muscle during transition from substrate-free perfusion to perfusion with glucose plus insulin (19).

The possible physiological relevance of the accumulation of succinate is not clear. There are no known extra-mitochondrial pathways in succinate metabolism. In rat heart mitochondria incubated with pyruvate also accumulation of succinate takes place (1-4), whereas no accumulation of succinate was reported during perfusion of rat heart with glucose plus insulin (19).

4.4.2. *Measurement of citric acid cycle activity with ^{14}C-labeled substrates*

Information concerning citric acid cycle activity during pyruvate plus malate oxidation in muscle mitochondria can also be obtained by measuring the rate of $^{14}CO_2$ production from ^{14}C-labeled pyruvate or malate. When using [2-^{14}C]pyruvate as substrate, $^{14}CO_2$ is liberated after one full turn of the cycle (20). This means that ^{14}C is stored in accumulated 2-oxoglutarate and succinate and is subject to considerable label dilution by the large pool of unlabeled

malate before any $^{14}CO_2$ is liberated. When using [U-^{14}C]malate as substrate, $^{14}CO_2$ is liberated during the first turn of the cycle. Mixing of the [U-^{14}C] malate pool with citric acid cycle derived malate can be prevented by addition of malonate. On base of these considerations citric acid cycle activity was measured with [U-^{14}C]malate as labeled substrate.

The decrease in $^{14}CO_2$ production from [1-^{14}C]pyruvate by malonate may be explained by competition of malate and malonate for transport by the dicarboxylate translocase (13). This will result in a decrease in the amount of malate transported and hence the effective malate concentration will be lowered, resulting in a decreased stimulation of pyruvate oxidation.

The dissimilar rates of $^{14}CO_2$ production from [U-^{14}C]malate with pyruvate and acetylcarnitine in rat muscle mitochondria (Table 4.1) indicate that the pyruvate dehydrogenase activity is higher than the carnitine acetyltransferase activity. In human muscle mitochondria these activities appear to be about equal.

The divergence between the activity of pyruvate dehydrogenase and isocitrate dehydrogenase in human muscle mitochondria could not be explained by accumulation of citrate. Such an accumulation is not likely since muscle mitochondria do not possess a tricarboxylate translocase (18). Furthermore, in human muscle mitochondria citrate synthase and isocitrate dehydrogenase activities are about equal (21). It seems therefore that part of the acetylCoA formed by the pyruvate dehydrogenase complex is converted into acetate, as was found with mitochondria from rat heart (1,22,23) and house fly flight muscle (24).

Parallel incubations of human muscle mitochondria with [U-^{14}C]malate and either pyruvate or acetylcarnitine will be the best means of obtaining information concerning citric acid cycle activity in the span between entry of acetylCoA and 2-oxoglutarate oxidation, since acetylCoA is supplied through two independent pathways. If these incubations are performed differentiation will be possible between a defect in pyruvate dehydrogenase, carnitine acetyltransferase and the first segment of the citric acid cycle. The activity of the second segment of the cycle, the oxidation of 2-oxoglutarate to succinate, can be measured with [1-^{14}C]2-oxoglutarate as substrate. The activity of the enzymes of the last segment cannot be measured by the rate of $^{14}CO_2$ production from labeled substrates since there are no decarboxylation steps involved in the oxidation of succinate to malate. However, the stimulation of the rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate by succinate or fumarate (Chapter 3) may be taken as a measure of the activities of the enzymes of this last segment of

the citric acid cycle.

4.5. SUMMARY

Citric acid cycle activity was investigated in skeletal muscle mitochondria oxidizing pyruvate plus malate. The accumulation of cycle intermediates indicates that the citric acid cycle operates in three segments: entry of acetylCoA to 2-oxoglutarate, 2-oxoglutarate to succinate and succinate to malate.
By reconstitution of the malate-aspartate shuttle the accumulation of 2-oxoglutarate is eliminated and glutamate accumulates.
Due to the accumulation of cycle intermediates overall flux through the citric acid cycle cannot be measured in skeletal muscle by determining the rate of $^{14}CO_2$ production from ^{14}C-labeled substrates. The activity of the first segment of the cycle can be measured with [U-^{14}C]malate as substrate. Oxidation of 2-oxoglutarate to succinate can be assayed with [1-^{14}C] 2-oxoglutarate. The stimulation of $^{14}CO_2$ production from [1-^{14}C]pyruvate in the presence of succinate or fumarate gives an indication of the activities of the enzymes of the last segment of the citric acid cycle.

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CHAPTER 5

RECONSTITUTION OF MALATE-ASPARTATE AND α-GLYCEROPHOSPHATE SHUTTLE ACTIVITY IN RAT SKELETAL MUSCLE MITOCHONDRIA

5.1. INTRODUCTION

The inner mitochondrial membrane is impermeable for NADH (1). Therefore, reducing equivalents generated in the cytosol must be transported across the inner mitochondrial membrane in some other form before they can be oxidized by the mitochondrial electron transport chain. A number of shuttle mechanisms have been postulated to carry out this process by transporting reducing equivalents across the mitochondrial membrane in the form of a reduced substrate which is oxidized within the mitochondrion and then transported back to the cytosol to become reduced again (2-4). Among these shuttles the malate-aspartate shuttle and the α-glycerophosphate shuttle are thought to be the most important ones (5). Operation of the α-glycerophosphate shuttle does not actually involve substrate translocation across the inner mitochondrial membrane since the enzyme α-glycerophosphate dehydrogenase is located on the outer site of the mitochondrial inner membrane (6).
In this chapter the transport of reducing equivalents across the mitochondrial membrane of rat skeletal muscle mitochondria was studied. The malate-aspartate shuttle and the α-glycerophosphate shuttle were reconstituted by addition of the cytosolic components of these shuttle systems to the incubation medium. Shuttle activities were assayed by measuring the rate of NAD^+ formation from added NADH. The effect of respiratory chain inhibitors and uncoupler as well as the effect of citric acid cycle inhibitors on the activity of the reconstituted shuttles was investigated.

5.2. MATERIALS AND METHODS

Rat skeletal muscle mitochondria were isolated as described in Chapter 3. Incubations were performed in the basic incubation medium, final volume 1 ml. In those incubations to which FCCP was added, ADP and hexokinase were omitted. Incubations were terminated after 10 or 15 min by addition of 0.2 ml 6 M $HClO_4$.
Reconstitution of malate-aspartate shuttle was performed by addition of 10 mM

glutamate, 2 mM malate, 5 mM aspartate, 5 units glutamate-oxaloacetate transaminase and 12 units malate dehydrogenase. The α-glycerophosphate shuttle was reconstituted by addition of 10 mM L-glycerol-3-phosphate and 3 units α-glycerophosphate dehydrogenase. Initial NADH concentration was 5 mM with both reconstituted shuttles. Under all conditions tested, incubations were also performed in the absence of reconstituted shuttle systems to correct for NADH oxidation which was not linked to shuttle activity.
Glutamate-oxaloacetate transaminase, α-glycerophosphate dehydrogenase and malate dehydrogenase were obtained from Boehringer, Mannheim, W-Germany and were dialyzed against 20 mM potassium phosphate buffer, containing 1 mM EDTA, pH 6.7, to remove ammonium sulphate present in these commercial preparations. NAD^+ and glucose-6-phosphate were assayed in neutralized perchloric acid samples of the incubation medium with alcoholdehydrogenase and ethanol (7) and with $NADP^+$ and glucose-6-phosphate dehydrogenase (8), respectively.

5.3. RESULTS

5.3.1. *Effect of respiratory chain inhibitors and uncoupler on shuttle activities*

The activity of the reconstituted malate-aspartate shuttle and α-glycerophosphate shuttle was determined by measuring the rate of formation of NAD^+ from NADH. Preliminary experiments showed that with the concentrations of glutamate, malate, aspartate and α-glycerophosphate used, maximal rates of NAD^+ formation were obtained with the respective shuttles. Under the conditions employed the rate of NAD^+ formation was linear with respect to time and amount of protein present with both reconstituted shuttle systems (Fig. 5.1). The rate of NAD^+ formation by the reconstituted malate-aspartate shuttle is about 50 % greater than that observed with the reconstituted α-glycerophosphate shuttle (Table 5.1). Simultaneous reconstitution of both shuttle systems results in a rate of NAD^+ formation which is the sum of the rates obtained with the separate shuttles. The activity of the malate-aspartate shuttle is completely blocked by addition of rotenone, whereas no effect on α-glycerophosphate shuttle activity is observed. Antimycin A inhibits both reconstituted shuttles. The inhibition of malate-aspartate shuttle activity by FCCP indicates that this shuttle system is dependent on energy supply, which may be explained by the energy linked efflux of aspartate from the mitochondria (9-12).

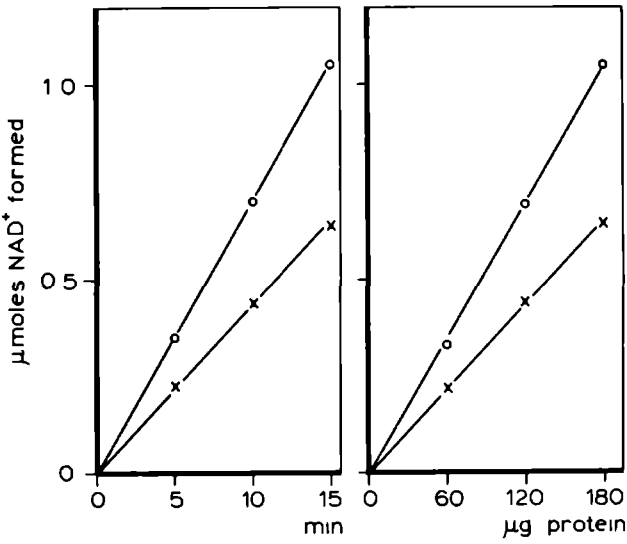

Fig. 5.1. Rate of NAD^+ formation by the reconstituted malate-aspartate (o——o) and α-glycerophosphate (x——x) shuttle as a function of time (mitochondrial protein concentration 180 µg/ml) and as a function of the amount of mitochondrial protein (incubation time 15 min). Data were taken from one representative experiment.

5.3.2. *Stoichiometry between NAD^+ and ATP formation*

To assay the stoichiometry between shuttle mediated NADH oxidation and ATP formation, glucose-6-phosphate formation was measured in addition to NAD^+ formation. The ratio between glucose-6-phosphate and NAD^+ formation by the α-glycerophosphate shuttle was 1 20 ± 0.06 (mean ± S.D , n=3). A (theoretically impossible) ratio of 4.55 for NADH oxidation was obtained with the malate-aspartate shuttle (Table 5.2). This may be explained by assuming that not only extra-mitochondrial NADH but also intra-mitochondrially generated NADH is oxidized. To investigate this possibility, the effect of inhibitors of citric acid cycle activity was tested. Addition of arsenite to the incubation medium lowered the rate of glucose-6-phosphate production and increased the rate of NAD^+ formation, resulting in a ratio between glucose-6-phosphate and NAD^+ formation of 1.95. The mean value obtained under this condition was 2.03 ± 0.15 (mean ± S.D., n=3). Addition of malonate did not influence the rates of

TABLE 5.1.

EFFECT OF RESPIRATORY CHAIN INHIBITORS AND UNCOUPLER ON SHUTTLE ACTIVITIES

Rat skeletal muscle mitochondria (final concentration about 0.1 mg protein/ml) were incubated in the medium described under Materials and Methods. Inhibitor concentrations were 1 μg/ml rotenone, 1 μg/ml antimycin A and 2 mM KCN. Final concentration of FCCP was 0.2 μM. Values are the means $\pm$ S.D. of three or four separate experiments.

Additions	Shuttle activity (μmol NAD^+ formed/mg protein.h)		
	Malate-aspartate	α-glycerophosphate	Malate-aspartate + α-glycerophosphate
None	20.2 $\pm$ 3.0	13.0 $\pm$ 2.2	31.4 $\pm$ 4.3
Rotenone	<1	13.3 $\pm$ 1.9	13.7 $\pm$ 1.7
Antimycin A	<1	<1	<1
FCCP	<1	14.8 $\pm$ 2.2	21.8 $\pm$ 3.7

TABLE 5.2.

STOICHIOMETRY BETWEEN THE FORMATION OF GLUCOSE-6-PHOSPHATE AND NAD^+ BY THE RECONSTITUTED MALATE-ASPARTATE SHUTTLE

Rat skeletal muscle mitochondria (final concentration 0.11 mg protein/ml) were incubated in the medium as described under Materials and Methods. Where indicated arsenite and malonate were added in a concentration of 2 mM and 5 mM, respectively.

Addition	Glucose-6-phosphate (μmol/mg protein.h)	NAD^+	Glucose-6-phosphate/NAD^+ ratio
None	119.2	26.2	4.55
Arsenite	67.4	34.6	1.95
Malonate	119.7	26.1	4.59

NAD^+ and glucose-6-phosphate formation.

5.3.3. *Interaction between malate-aspartate and α-glycerophosphate shuttles in the presence of uncoupler*

In the presence of FCCP, simultaneous reconstitution of both shuttles shows a rate of NAD^+ formation which exceeds the sum of the rates measured with the separate shuttles (Table 5.1). To investigate whether this increment in NAD^+ formation was due to an increase in activity of the malate-aspartate shuttle, of the α-glycerophosphate shuttle or of both shuttles, the experiment presented in Table 5.3 was performed. It clearly shows that the rate of NAD^+ formation in the presence of FCCP and both shuttles is reduced by addition of rotenone to the rate measured with the α-glycerophosphate shuttle separately, which indicates that the increment in NAD^+ formation originates from malate-aspartate shuttle activity.

TABLE 5.3.

EFFECT OF ROTENONE ON SHUTTLE ACTIVITIES IN UNCOUPLED MITOCHONDRIA

Rat skeletal muscle mitochondria (final concentration 0.13 mg protein/ml) were incubated in the medium described under Materials and Methods supplemented with 0.2 μM FCCP. Rotenone (1 μg/ml) was added as indicated. Activities of the shuttle systems are given in μmol NAD^+ formed/mg protein.h.

Shuttle system	- rotenone	+ rotenone
Malate-aspartate	<1	<1
α-Glycerophosphate	16.9	16.3
Malate-aspartate + α-glycerophosphate	23.9	16.4

5.4. DISCUSSION

In the present study, rat skeletal muscle mitochondria were incubated with a high concentration of NADH to measure the activity of the reconstituted malate-aspartate and α-glycerophosphate shuttle. Previous investigations were performed with NADH regenerating systems containing NAD^+ and either lactate plus lactate dehydrogenase (9) or ethanol plus alcohol dehydrogenase (13). The former system has a low capacity to regenerate NADH, and some of the pyruvate formed may undergo further oxidation through the citric acid cycle, thereby reducing the amount of oxaloacetate available for malate-aspartate shuttle activity. The latter system suffers from the disadvantage that under conditions where respiratory chain inhibitors are used acetaldehyde accumulates in the incubation medium which subsequently inhibits alcoholdehydrogenase activity (14). These problems are circumvented by incubation with NADH. The linear rate of NAD^+ production measured with respect to the amount of protein indicates that the rate of NAD^+ formation is determined by the activity of the mitochondria.

The effect of rotenone on shuttle activities (Table 5.1) indicates that NADH oxidation mediated by the malate-aspartate shuttle proceeds through the NADH dehydrogenase region of the respiratory chain whereas reducing equivalents transported by the α-glycerophosphate shuttle enter the respiratory chain distal to the rotenone block. The latter is in contrast to the inhibition of α-glycerophosphate shuttle activity by rotenone in rat liver mitochondria observed by Cederbaum et al. (13). However, those experiments were performed with a NADH regenerating system using alcoholdehydrogenase.

Inhibition of 2-oxoglutarate dehydrogenase by arsenite has a dual effect on the stoichiometry of the malate-aspartate shuttle: the rate of NAD^+ formation increases and the rate of glucose-6-phosphate formation decreases. No effect is observed by the inhibition of succinate dehydrogenase by malonate (Table 5.2). This means that the presence of arsenite prevents that part of the 2-oxoglutarate generated intra-mitochondrially by the glutamate-oxaloacetate transaminase is oxidized to succinate. This results in an increased amount of 2-oxoglutarate available for shuttle activity and reduces the amount of ATP formed from oxidation of intra-mitochondrial NADH.

Uncoupling is known to result in an inhibition of aspartate efflux from mitochondria (9-12). This efflux could be restored in heart muscle mitochondria by addition of ATP and was oligomycin sensitive under this condition which

indicates that glutamate-aspartate exchange requires energization of the mitochondrial membrane (10). In our experiments a mean value of about 2 was found for the ratio between glucose-6-phosphate and NAD^+ formation during malate-aspartate shuttle activity in the presence of arsenite, indicating that for each molecule of NADH oxidized and hence for each molecule of extra-mitochondrial glutamate exchanged for intra-mitochondrial aspartate one molecule of ATP is needed. Simultaneous reconstitution of both shuttles in uncoupled rat skeletal muscle mitochondria results in a partial restoration of malate-aspartate shuttle activity (Table 5.3) of about 7 μmol NADH oxidized per mg protein.h. This activity needs a supply of 7 μmol ATP/mg protein.h. which can only be supplied by α-glycerophosphate shuttle activity. Since the latter proceeds at a mean rate of about 13 μmol/mg protein.h. and in the absence of FCCP with a ratio between glucose-6-phosphate and NAD^+ formation of 1.20, it can be calculated that about 40 % of the energy arising from α-glycerophosphate shuttle activity is not wasted in the presence of uncoupler but used to support malate-aspartate shuttle activity. Further investigations are needed to elucidate the mechanism which enables the glutamate-aspartate translocase to perform energy-linked transport in the presence of uncoupler and the reconstituted α-glycerophosphate shuttle.

5.5. SUMMARY

In this chapter transport of reducing equivalents across the mitochondrial membrane of skeletal muscle mitochondria was studied. Rat skeletal muscle mitochondria were incubated with NADH and the cytosolic components of the malate-aspartate shuttle or the α-glycerophosphate shuttle. The capacity for NADH oxidation was about 50 % higher with the malate-aspartate shuttle than with the α-glycerophosphate shuttle. During malate-aspartate shuttle activity considerable amounts of succinate are formed from 2-oxoglutarate, which is generated intra-mitochondrially through the glutamate-oxaloacetate transaminase. This reduces the amount of 2-oxoglutarate available for shuttle activity and limits the rate of oxidation of extra-mitochondrial NADH. Furthermore, a hitherto unknown interaction between the malate-aspartate and the α-glycerophosphate shuttle was found, which decreases the inhibition of malate-aspartate shuttle activity caused by uncoupler.

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CHAPTER 6

MEASUREMENT OF CYTOCHROMES IN HUMAN SKELETAL MUSCLE MITOCHONDRIA ISOLATED FROM FRESH AND FROZEN-STORED MUSCLE SPECIMENS

6.1. INTRODUCTION

Measurement of cytochromes in human skeletal muscle mitochondria may be of diagnostic value in the case of patients suffering from a "mitochondrial myopathy". However, few reports appeared in the literature concerning cytochromes in human skeletal muscle mitochondria.

Makinen and Lee (1) and DiMauro et al. (2) measured difference spectra of human skeletal muscle mitochondria at the temperature of liquid nitrogen. The absolute amount of cytochromes was calculated after correction for the increase in absorbance due to the low temperature. This correction was based on data obtained from experiments with rat and canine skeletal muscle mitochondria (1).

Spiro et al. (3, 4) measured cytochromes in human skeletal muscle mitochondria at room temperature. However, they reported the ratio between the different cytochromes but not the absolute amount. A decrease in cytochrome content in human muscle mitochondria was reported by several authors (4-9).

In this study, human skeletal muscle mitochondria were isolated from small muscle biopsy specimens. Difference spectra of cytochromes were recorded at room temperature after reduction of the cytochromes with dithionite or with succinate plus KCN. The latter method was used to avoid reduction of hemoglobin or myoglobin which was always present in amounts which caused a heavy contamination of the spectra. Also, muscle specimens were stored at -70^{o} prior to the isolation of mitochondria and the subsequent measurement of the cytochrome spectra. The effect of storage of muscle specimens at -70^{o} on the cytochrome content finally present in the mitochondria was investigated.

6.2. MATERIALS AND METHODS

Human skeletal muscle specimens (1.5-2.0 g) were obtained from patients with no known history of neuromuscular disease. Rat skeletal muscle was obtained from the hind leg of male Wistar rats, weighing 150-200 g. The muscle specimens were placed immediately in an ice-cold medium, containing 250 mM sucrose,

2 mM EDTA, 10 mM Tris-HCl and 50 U/ml heparin, pH 7.4 (SETH-medium), or were stored at -70°. Mitochondria were isolated from fresh and from stored muscle tissue (after thawing) as described in chapter 3.

Spectra. Difference spectra between the reduced and the oxidized forms of the cytochromes were recorded at room temperature on a Cary 118C double-beam recording spectrophotometer. Both the reference and the sample cuvette (optical pathway 10 mm) were filled with a suspension containing 0.3-0.6 mg mitochondrial protein per ml, 24 mM potassium phosphate, 2 mM EDTA, 10 mM Tris-HCl, 60 mM KCl, 7 mM $MgCl_2$, 90 mM sucrose, 1.6 mM ADP, final pH 7.4. Under this condition the mitochondria are in the fully oxidized state or State 2 (10).

Reduction of the cytochromes in the sample cuvette was achieved by one of the following two methods:

a. 10 mM succinate plus 1 μg antimycin A was added to the mitochondrial suspension in the sample cuvette and the spectrum was recorded. Hereafter, a few grains of dithionite were added and the spectrum was recorded again.
b. 10 mM succinate plus 1 mM KCN was added to the sample and the spectrum was recorded.

For calculation of the mitochondrial cytochrome content the following extinction coefficients were used: cytochrome aa_3 $\Delta\epsilon_{605-630} = 16.5\ mM^{-1}$ (11), cytochrome b $\Delta\epsilon_{565-575} = 17.9\ mM^{-1}$ (12) and cytochrome $c+c_1$ $\Delta\epsilon_{550-540} = 19.0\ mM^{-1}$ (13).

Protein was determined by the method of Lowry et al[14]. Statistical analyses were performed with the Student's t test.

6.3. RESULTS

6.3.1. *Spectra*

Fig. 6.1 shows the difference spectrum of human skeletal muscle mitochondria. After reduction with succinate plus antimycin A the spectrum of cytochrome b is recorded (middle curve). The α-band of cytochrome b has its maximum at 565 nm, whereas the γ-band can be seen at 431 nm. Subsequent addition of dithionite results in reduction of all the cytochromes as shown in the upper curve. The α-bands of cytochrome aa_3 and cytochrome $c+c_1$ have their maxima at 606 nm and 551 nm, respectively. The γ-band of cytochrome aa_3 has its maximum at 445 nm. Subtraction of the spectrum of cytochrome b from the spectrum obtained after dithionite reduction gives the spectrum of cytochrome aa_3 and $c+c_1$

(lower curve).

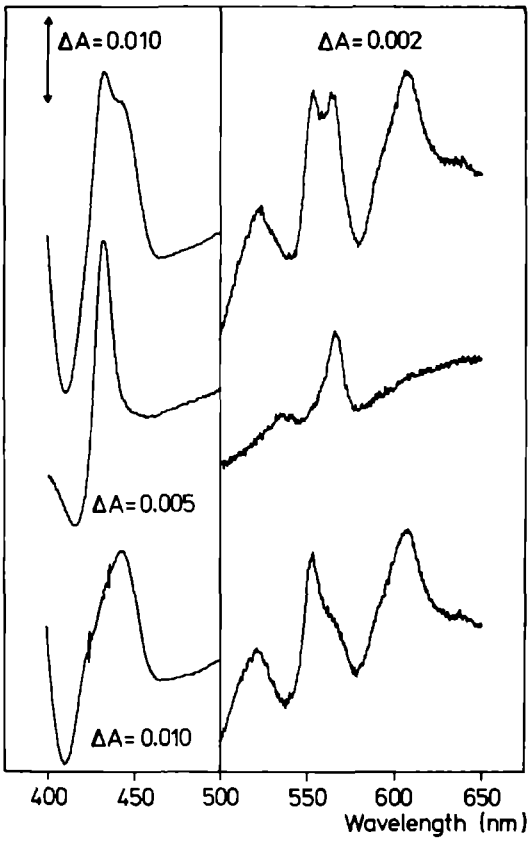

Fig. 6.1. Difference spectrum of human skeletal muscle mitochondria. After addition of succinate plus antimycin A to the sample cuvette the spectrum of cytochrome b is recorded (middle curve). Subsequent addition of dithionite reduces all the cytochromes (upper curve). The lower curve shows the difference spectrum obtained after reducing the mitochondrial suspension in the sample cuvette with succinate plus antimycin A followed by dithionite and the suspension in the reference cuvette with succinate plus antimycin A. For further experimental details see text.

In Fig. 6.2 the spectrum of rat skeletal muscle mitochondria is shown. Comparing these spectra (Figs. 6.1 and 6.2) it can be seen that addition of dithionite to the human mitochondrial suspension results in an increase in the

absorbance difference between the peak at 565 nm and the trough at 575 nm. Moreover, there is a marked increase in the absorbance at 431 nm which does not allow resolution of the γ-band of cytochrome $c+c_1$ at 420 nm (lower curves).

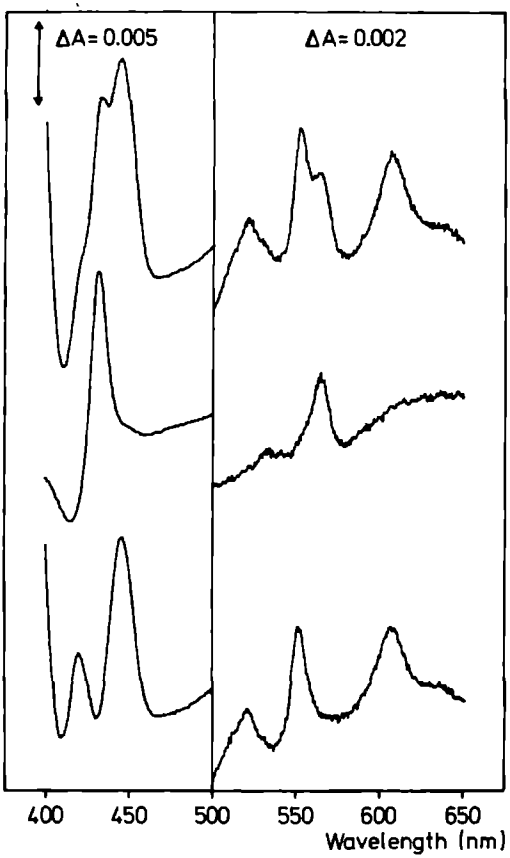

Fig. 6.2. Difference spectrum of rat skeletal muscle mitochondria. The assay conditions were the same as described for Fig. 6.1.

This indicates contamination of the mitochondrial suspension with hemoglobin (Hb) or myoglobin (Mb). The dithionite reduced minus oxidized difference spectra of human Hb and Mb are shown in Fig. 6.3. Since these difference spectra of human Hb and Mb are very similar it is not possible to distinguish between spectra from Hb contaminated and Mb contaminated mitochondrial suspensions. It can be seen in Fig. 6.1 that this contamination mostly affects

the α- and γ-band of cytochrome b, which is the reason that cytochrome b was measured separately with succinate plus antimycin A. By reducing the cytochromes with succinate plus KCN instead of dithionite the problem of contamination is eliminated since there is no reduction of Hb and Mb under this condition.

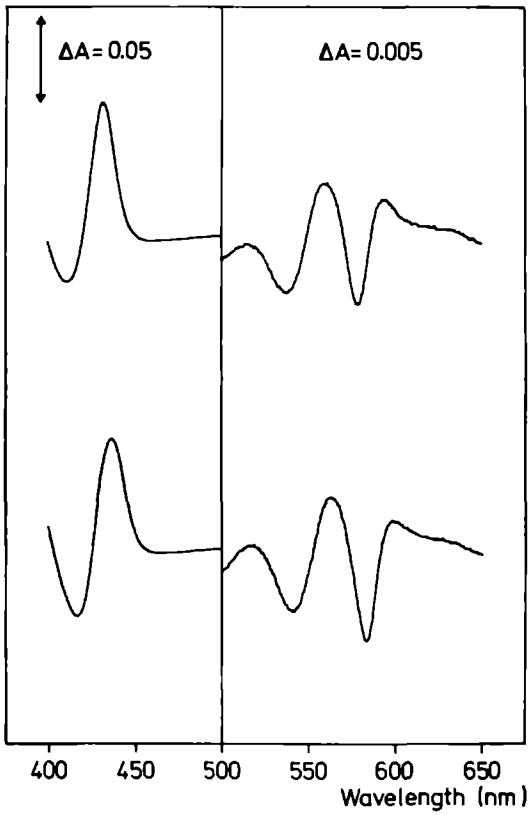

Fig. 6.3. Difference spectrum of human hemoglobin (upper curve) and human myoglobin (lower curve).
Reduction was achieved by addition of dithionite.

6.3.2. *Cytochrome content of human skeletal muscle mitochondria*

The cytochrome content of human skeletal muscle mitochondria is presented in Table 6.1. The mean values obtained after reduction with succinate plus KCN are lower than those obtained after succinate plus antimycin A followed by dithionite reduction. The difference is significant ($P<0.05$) in the case of

cytochromes b and $c+c_1$. Although the mean value obtained for cytochrome aa_3 in the presence of succinate plus KCN is lower than in the presence of dithionite, statistical analysis shows this difference to be not significant ($P > 0.05$).

TABLE 6.1.

CYTOCHROME CONTENT OF HUMAN SKELETAL MUSCLE MITOCHONDRIA

Values shown are the means ± SD of the number of experiments shown in the parentheses.
n.s.: not significant

Reduction Method	aa_3	b	$c+c_1$	aa_3	b	$c+c_1$
	pmoles/mg protein			relative amount		
Succinate + Antimycin A/ dithionite (n=5)	444 ± 66	423 ± 65	906 ± 187	1.0	0.95	2.04
Succinate + KCN (n=8)	374 ± 117	290 ± 71	648 ± 187	1.0	0.78	1.73
P	n.s.	< 0.01	< 0.05			

It is not always possible to perform measurement of cytochromes immediately after taking the muscle biopsy specimen. Therefore, we investigated the effect of storage of muscle tissue on the content of cytochromes. Muscle tissue was stored for periods up to three months at -70^{o}. After thawing, mitochondria were isolated and cytochromes were measured exactly as described for fresh muscle specimens. The cytochrome content of these mitochondria is presented in Table 6.2. The contamination in mitochondrial suspensions obtained from muscle tissue which has been stored at -70^{o} was considerably greater as compared with mitochondrial suspensions obtained from fresh muscle tissue.

TABLE 6.2.

CYTOCHROME CONTENT OF HUMAN SKELETAL MUSCLE MITOCHONDRIA ISOLATED FROM MUSCLE TISSUE STORED AT -70°

Values shown are the means ± SD of the number of experiments shown in the parentheses.

Reduction Method	aa_3	b	$c+c_1$	aa_3	b	$c+c_1$
	pmoles/mg protein			relative amount		
Succinate + Antimycin A/ dithionite (n=4)	450 ± 52	488 ± 107	-	1.0	1.08	-
Succinate + KCN (n=4)	305 ± 28	241 ± 9	414 ± 79	1.0	0.79	1.36
P	<0.01	<0.05	-			

Due to this contamination the cytochrome $c+c_1$ peak is poorly resolved after dithionite addition and a reliable calculation of the cytochrome $c+c_1$ content was impossible. Comparison of the cytochrome values obtained after reduction with succinate plus KCN with those obtained after reduction with succinate plus antimycin A, followed by dithionite reveals again lower values for cytochrome aa_3 and cytochrome b in the former method, as was the case with fresh muscle. In contrast to mitochondria isolated from fresh muscle specimens, the difference in values found for cytochrome aa_3 is now significant.
Comparison of the values obtained with succinate plus KCN in fresh and -70° stored muscle shows a decrease in the relative amount of cytochrome $c+c_1$ after storage (Table 6.1 and 6.2).

6.4. DISCUSSION

Measurement of cytochrome spectra from human skeletal muscle mitochondria by means of dithionite reduced minus oxidized difference spectrophotometry showed that the mitochondrial suspension is contaminated with Hb or Mb, in contrast to mitochondria isolated from rat skeletal muscle. This contamination could not be removed by extensive washing of the mitochondrial fraction during the isolation procedure. Contamination of mitochondrial suspensions with Hb has also been described for human liver mitochondria (15). Reduction with succinate plus KCN instead of dithionite eliminates the problem of contamination from the spectra.

Significantly higher values were found for cytochrome b and cytochrome $c+c_1$ after reduction with succinate plus antimycin A followed by addition of dithionite as compared with the values found after reduction with succinate plus KCN, in contrast to the values found for cytochrome aa_3 by these methods. An explanation for this phenomenon that higher amounts of cytochromes are found after reduction with dithionite might be that not all of the cytochromes are linked to the respiratory chain, as has been concluded by Sato and Hagihara for cytochromes in mouse ascites hepatoma cells (16). An alternative explanation may be that the degree of reduction of the cytochromes and thus the amount of cytochromes calculated to be present is dependent on the method of reduction. This latter explanation is certainly true in the case of cytochrome b. It can be observed (see Tables 6.1 and 6.2) that a much higher amount of cytochrome b is measured in the presence of succinate plus antimycin A than in the presence of succinate plus KCN. Storage of muscle tissue at -70^{o} does not result in a loss of cytochrome b and cytochrome aa_3 from the mitochondria as can be concluded from the values found in fresh and stored muscle after reduction of the cytochromes with succinate plus antimycin A followed by dithionite (Tables 6.1 and 6.2). The values found after reduction with succinate plus KCN in stored muscle are lower than those obtained with dithionite, as was the case with fresh muscle. The difference in values found for cytochrome aa_3 in mitochondria isolated from stored muscle specimens is significant in contrast to the difference found with mitochondria isolated from fresh muscle tissue. Comparison of the relative amount of cytochromes found after reduction with succinate plus KCN in mitochondria isolated from fresh or stored muscle tissue reveals a difference in stoichiometry which clearly points at a loss of cytochrome $c+c_1$ from the mitochondria after storage of the

muscle tissue. This may be caused by the much weaker binding of cytochrome c to the mitochondrial membrane as compared with the other cytochromes.
In our opinion measurement of difference spectra of cytochromes is a valuable diagnostic tool in the field of muscle disease, especially since its use is not restricted to mitochondria isolated from fresh muscle biopsy specimens, but it can also be applied to muscle tissue which has been stored at -70^{o}. Since preparations of human skeletal muscle mitochondria are contaminated with Hb or Mb, the use of dithionite as reductant leads to contaminated spectra which can easily be misinterpreted (4) and should therefore be avoided.

6.5. SUMMARY

Mitochondria were isolated from small muscle biopsy specimens, and the cytochrome content was calculated from the reduced minus oxidized difference spectrum recorded at room temperature.
From the difference spectra obtained after reduction of the cytochromes with dithionite it is concluded that human mitochondrial suspensions are contaminated with hemoglobin or myoglobin.
The cytochrome content calculated after reduction of the cytochromes with succinate plus KCN is lower than that obtained after reduction with dithionite, indicating incomplete reduction of the cytochromes by the former method. Storage of muscle tissue at -70^{o} before isolation of mitochondria results in a loss of cytochrome $c+c_1$ from these mitochondria.

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CHAPTER 7

THE EFFECT OF EXOGENOUS CYTOCHROME C ON PYRUVATE OXIDATION BY RAT SKELETAL MUSCLE MITOCHONDRIA ISOLATED IN SUCROSE OR KCL MEDIUM

7.1. INTRODUCTION

Cytochrome c is readily released from the mitochondrial membrane by washing mitochondria with hypotonic KCl solutions (1), treatment of mitochondria with digitonin (2) or sonification of mitochondrial suspensions during preparation of submitochondrial particles (3).
These results indicate the more labile binding of cytochrome c to the mitochondrial membrane as compared with the other cytochromes.
Loss of cytochrome c from mitochondria results in a diminished oxidative capacity of the mitochondria which can be restored upon addition of cytochrome c (4, 5).
In a recent report, Van Handel et al. (6) described a stimulation by exogenous cytochrome c on the rate of pyruvate oxidation by rat heart and skeletal muscle mitochondria isolated in an isotonic KCl medium. The authors suggested that previous studies in which cytochrome c was not included in the assay medium have underestimated the respiratory capacity of the mitochondria.
The experiments described in this chapter were designed to evaluate the suggestion that addition of cytochrome c compensates for cytochrome c lost during isolation. If this suggestion is true, a correlation must be present between the stimulation of pyruvate oxidation by exogenous cytochrome c and the cytochrome c content of the mitochondria after isolation. To vary the amount of cytochrome c skeletal muscle mitochondria were isolated in three different isolation media: isotonic sucrose-, isotonic KCl- and hypotonic sucrose. The effect of these different isolation media on morphology, specific activity of cytochrome oxidase, respiratory control index, P/O ratio and cytochrome content was studied as well as the effect of exogenous cytochrome c on the rate of pyruvate oxidation by these mitochondria.
Possible relevance of the results for clinical investigations with human skeletal muscle mitochondria is discussed.

Isolation of skeletal muscle mitochondria. Skeletal muscle mitochondria were isolated from the hind limb of male Wistar rats, weighing 150-200 g and fasted overnight. All operations were carried out between 0 and 4°. Mitochondria were isolated in one of the following media:

a. isotonic sucrose. Muscle was freed from fat and connective tissue, cut into small pieces with scissors and suspended in a concentration of 1 g/10 ml in SETH medium, containing 250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl and 50 U/ml heparine, final pH 7.4. This muscle mince was homogenized in a Potter-Elvehjem homogenizer by hand. The homogenate was centrifuged twice for 10 min at 600 xg to remove nuclei and cell debris. Mitochondria were isolated by centrifugation for 10 min at 14000 xg. The mitochondrial pellet was rinsed and homogenized in SET medium, which is SETH medium from which heparine is omitted, and centrifuged again for 10 min at 14000 xg.
b. isotonic KCl. Mitochondria were isolated as described above except that the sucrose in the SETH medium was replaced by 125 mM KCl. After the first 14000 xg centrifugation step the mitochondrial pellet was resuspended in SET medium in which the mitochondria were also finally taken up.
c. hypotonic sucrose. Mitochondria were isolated as described above (a). After the first 14000 xg centrifugation step the mitochondrial pellet was rinsed and resuspended in SET medium which was diluted with distilled water to a final sucrose concentration of 50 mM. After centrifugation the mitochondria were finally taken up in SET medium.

Cytochromes. Cytochromes were measured by difference spectrophotometry as described in the previous chapter. Reduction was achieved by adding 10 mM succinate plus 1 mM KCN to the sample cuvette.

Oxygen consumption. Oxygen consumption was measured with 1 mM pyruvate and 1 mM malate as substrates at 37° in the medium described by Max et al. (7), which is a simplified version of the medium described by Dow (8). Respiratory control index and P/O ratio were calculated as described by Estabrook (9).

Pyruvate oxidation. The rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate was measured in the presence of 1 mM malate as described in chapter 3.

Electron microscopy. Mitochondrial pellets were fixed in 2 % glutaraldehyde, postfixed in 2 % osmium tetroxide, dehydrated with a graded series of ethanol and embedded in Epon 812. Ultrathin sections were double stained with uranyl-acetate and leadcitrate and examined on a Philips EM-300 electron microscope.

Cytochrome oxidase. Cytochrome oxidase activity was determined in a medium containing 50 mM potassium phosphate, pH 7.4 and approximately 70 μM reduced cytochrome c. The oxidation of cytochrome c was followed spectrophotometrically by monitoring the decrease in absorbance at 550 nm. Activity is expressed in units (μmol cytochrome c oxidized/mg protein min). The assay was performed at room temperature.
Protein was determined according to Lowry et al. (10).

7.3. RESULTS

7.3.1. *General characteristics*

Electron micrographs of skeletal muscle mitochondria isolated in isotonic sucrose, show an intact mitochondrial membrane and a regular arrangement of cristae (Fig. 7.1). The use of isotonic KCl leads to mitochondrial preparations which are less pure and homogenous. Mitochondria isolated in hypotonic sucrose show swelling of the mitochondrial matrix but the mitochondrial membrane appears intact. The purity of this preparation is comparable to that observed with mitochondria isolated in isotonic sucrose. The effect of different isolation media on protein yield and specific activity of cytochrome oxidase is shown in Table 7.1.

TABLE 7.1.

EFFECT OF DIFFERENT ISOLATION MEDIA ON PROTEIN YIELD AND CYTOCHROME OXIDASE ACTIVITY

Values shown are the means ± S.D. of the number of experiments shown in the parentheses.

Isolation medium	Protein (mg/g wet weight)	Cytochrome c oxidase (μmol/min. mg protein)
Isotonic sucrose	0.78 ± 0.21 (10)	8.1 ± 1.3 (6)
Isotonic KCl	0.93 ± 0.23 (5)	5.2 ± 0.7 (4)
Hypotonic sucrose	0.88 ± 0.05 (5)	8.4 ± 2.0 (4)

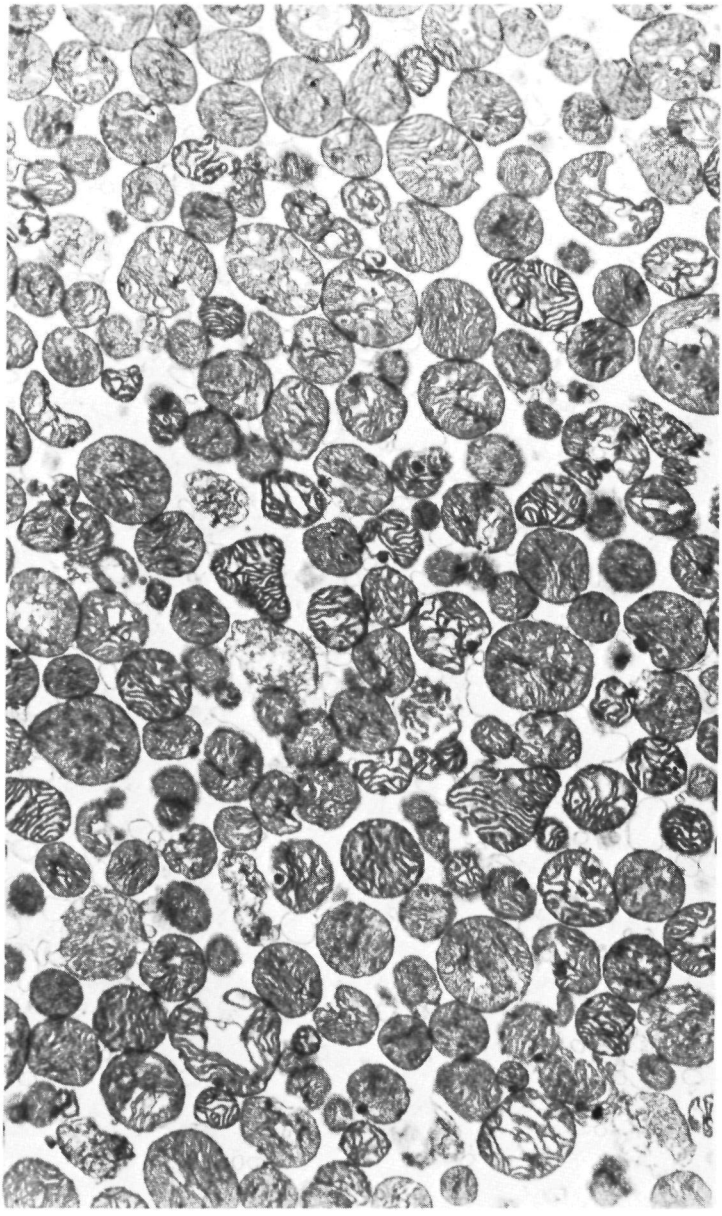

Fig. 7.1.A

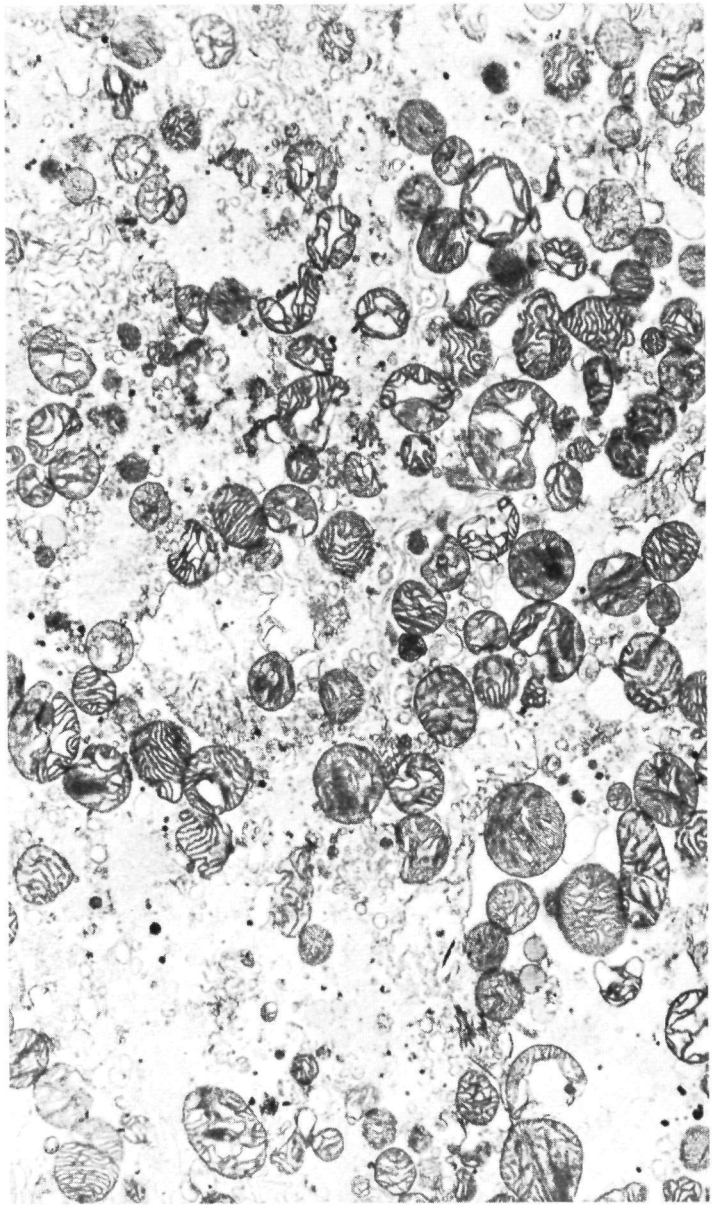

Fig. 7.1.B

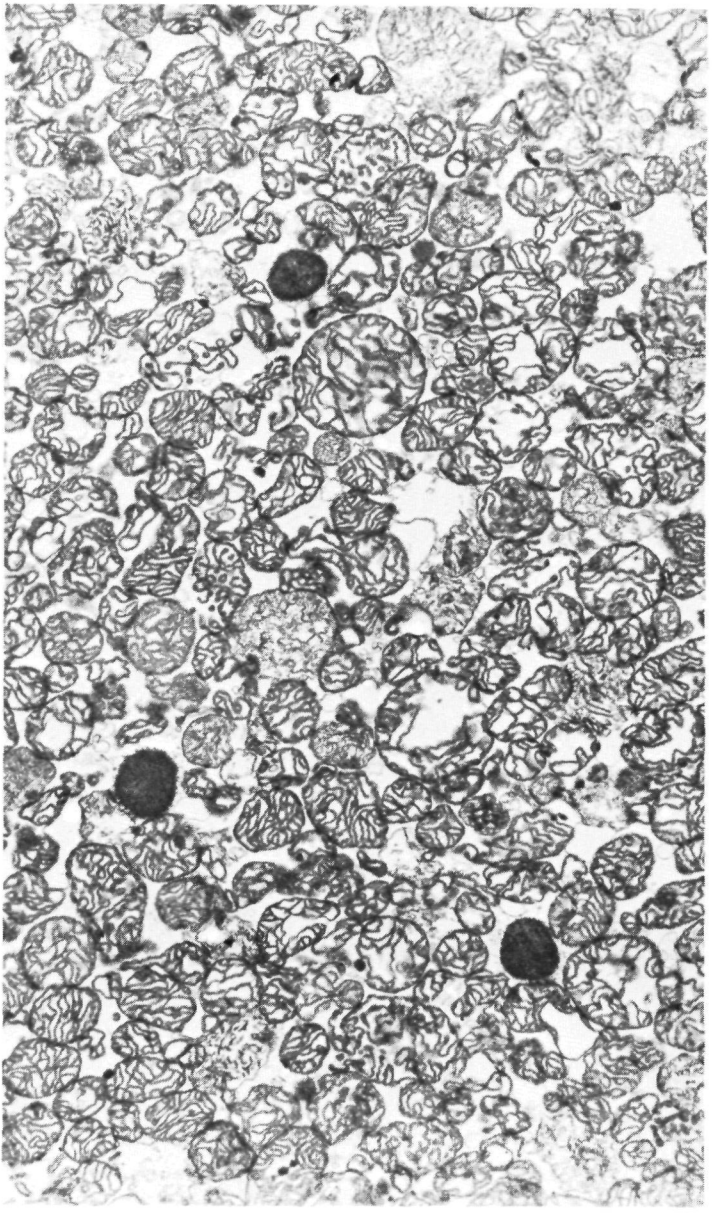

Fig. 7.1.C

Fig. 7.1. Electron micrographs of rat skeletal muscle mitochondria isolated in isotonic sucrose (A), isotonic KCl (B) and hypotonic sucrose (C). Magnification X 12,600.

The amount of protein obtained per g wet weight is similar in each medium, but mitochondria isolated in isotonic KCl have a lower specific activity of cytochrome oxidase. This indicates that the use of isotonic KCl leads to a mitochondrial preparation which is more contaminated with protein from non-mitochondrial origin. Furthermore, the total yield of cytochrome oxidase activity per g wet weight is lower in isotonic KCl isolated mitochondria which suggests a lower yield of mitochondria.

7.3.2. *Cytochrome content*

The use of isotonic KCl leads to a dramatic fall in the content of respiratory chain components of nearly 50 % as compared to the use of isotonic sucrose (Table 7.2). The ratio of the various cytochromes remains however fairly constant. Isolation of mitochondria in hypotonic sucrose has no effect on the levels of cytochrome aa_3 and b but leads to a preferential loss of cytochrome $c+c_1$ of about 20 %.

TABLE 7.2.

EFFECT OF DIFFERENT ISOLATION MEDIA ON CYTOCHROME CONTENT

Values shown are the means ± S.D. of the number of experiments given in the parentheses.

Isolation medium	aa_3	b	$c+c_1$	aa_3	b	$c+c_1$
	(pmol/mg protein)			(relative amount)		
Isotonic sucrose (9)	417 ± 29	366 ± 21	849 ± 82	1.0	0.88	2.04
Isotonic KCl (5)	226 ± 21	203 ± 21	437 ± 37	1.0	0.90	1.93
Hypotonic sucrose (5)	405 ± 14	386 ± 29	665 ± 43	1.0	0.95	1.64

7.3.3. *Effect of exogenous cytochrome c on electron transport*

Mitochondria isolated in sucrose are superior to mitochondria isolated in isotonic KCl with respect to respiratory control index and P/O ratio (Table 7.3).

TABLE 7.3.

EFFECT OF CYTOCHROME C ON RESPIRATORY CONTROL INDEX AND P/O RATIO

Values shown are the means ± S.D. of 8 (isotonic sucrose) or 5 (isotonic KCl, hypotonic sucrose) experiments.
Where indicated cytochrome c was added in a final concentration of 20 μM.

Isolation medium	Cytochrome c	RCI	P/O
Isotonic sucrose	-	6.7 ± 1.2	2.71 ± 0.11
	+	6.6 ± 1.0	2.70 ± 0.11
Isotonic KCl	-	4.5 ± 0.8	2.51 ± 0.23
	+	4.6 ± 0.7	2.51 ± 0.23
Hypotonic sucrose	-	5.8 ± 0.6	2.62 ± 0.05
	+	6.7 ± 0.6	2.60 ± 0.10

Exogenous cytochrome c has no effect on the respiratory control index of mitochondria isolated in isotonic sucrose or isotonic KCl. Addition of cytochrome c to mitochondria isolated in hypotonic sucrose increases the respiratory control index to the same value observed with mitochondria isolated in isotonic sucrose. No effect is observed after addition of cytochrome c on the P/O ratio with any of the isolation media, which indicates that exogenous cytochrome c does not interfere with the normal pathway of phosphorylation. Only under conditions where the pathway of electron transport via cytochrome oxidase is inhibited exogenous cytochrome c serves as terminal electron acceptor as appears from the following experiment (Fig. 7.2). Mitochondria were incubated with pyruvate and malate in the presence of cytochrome c and the reduction of cytochrome c was measured by monitoring the absorbance at 550 nm. No reduction of cytochrome c is observed unless a small amount of azide is added, which

causes partial inhibition of cytochrome oxidase (11). Subsequent addition of antimycin A in a concentration which completely blocks electron transport in the region between cytochrome b and cytochrome c_1 causes reoxidation of the previously reduced cytochrome c since under this condition transfer of electrons from cytochrome c via cytochrome oxidase to oxygen is the only mode of ATP synthesis.

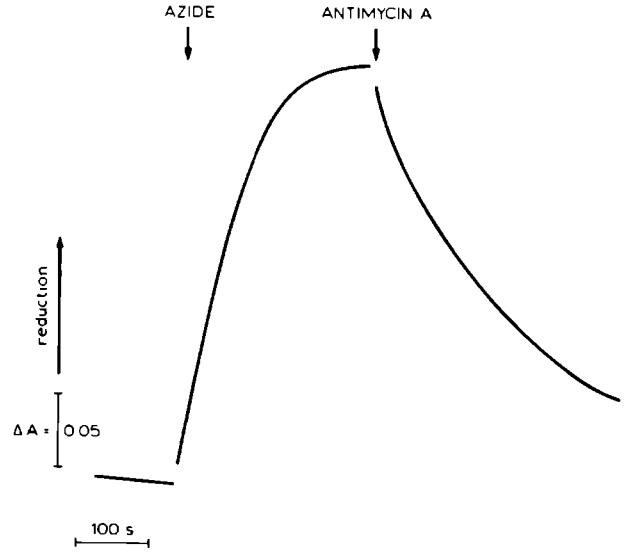

Fig. 7.2. Cytochrome c as terminal electron acceptor during pyruvate oxidation. Mitochondria (0.30 mg protein/ml) were incubated at room temperature in cuvettes in a final volume of 1 ml under the same conditions as employed for the assay of [1-^{14}C]pyruvate oxidation. The incubation medium was supplemented with 20 μM cytochrome c. Reduction of cytochrome c was measured by monitoring the absorbance at 550 nm. Sodium azide (1 mM) and antimycin A (1 μg) were added as indicated.

7.3.4. *Effect of exogenous cytochrome c on pyruvate oxidation*

Table 7.4 shows the effect of addition of cytochrome c on the capacity of skeletal muscle mitochondria to oxidize pyruvate, measured by the production of $^{14}CO_2$ from [1-^{14}C]pyruvate. The pyruvate oxidation rate expressed on base of protein found with mitochondria isolated in isotonic sucrose is twice the activity found with mitochondria isolated in the other two media. However, when the oxidation rates are expressed as μmol per unit cytochrome oxidase per

h or as nmol per pmol cytochrome aa_3 per h, the discrepancy between mitochondria isolated in isotonic sucrose and isotonic KCl disappears, while the oxidation capacity of mitochondria isolated in hypotonic sucrose still remains half of that found with mitochondria isolated in isotonic sucrose. Exogenous cytochrome c has a small stimulatory effect on the rate of pyruvate oxidation when added to mitochondria isolated either in isotonic sucrose or KCl. The stimulatory effect on mitochondria isolated in hypotonic sucrose is considerable and results in an almost complete restoration of pyruvate oxidation capacity on base of cytochrome oxidase activity and on base of spectrally measured cytochrome aa_3 when compared to mitochondria isolated in isotonic sucrose or KCl.

TABLE 7.4.

EFFECT OF CYTOCHROME C ON THE RATE OF [1-^{14}C]PYRUVATE OXIDATION

Values shown are the means ± S.D. of the number of experiments shown in the parentheses.
Where indicated cytochrome c was added in a final concentration of 20 μM.
cyt. ox.: cytochrome oxidase, cyt. aa_3: cytochrome aa_3.

Isolation medium	Cyto-chrome c	Pyruvate oxidation rate		
		μmol/mg. protein.h.	μmol/unit cyt. ox.h.	nmol/pmol cyt. aa_3.h.
Isotonic sucrose	-	15.7 ± 2.7 (10)	2.0 ± 0.5 (6)	36.3 ± 7.5 (10)
	+	18.7 ± 3.0 (10)	2.5 ± 0.6 (6)	43.0 ± 8.5 (10)
Isotonic KCl	-	7.4 ± 1.4 (5)	1.9 ± 0.7 (5)	33.2 ± 7.6 (5)
	+	9.9 ± 1.8 (5)	2.4 ± 0.8 (5)	42.0 ± 9.9 (5)
Hypotonic sucrose	-	7.3 ± 2.4 (5)	1.2 ± 0.3 (5)	18.2 ± 6.3 (5)
	+	15.7 ± 3.7 (5)	2.7 ± 0.9 (5)	39.0 ± 9.5 (5)

The rate of pyruvate oxidation (expressed on cytochrome oxidase base) in the absence of exogenous cytochrome c versus the ratio between cytochrome $c+c_1$ and cytochrome aa_3 shows a normal shaped v/s curve. The stimulation of pyruvate

oxidation rate observed after addition of cytochrome c is inversely related to the amount of cytochrome c present in the mitochondria after isolation. By plotting the logarithm of the ratio of the pyruvate oxidation rate in the presence and absence of added cytochrome c versus the ratio between cytochrome $c+c_1$ and cytochrome aa_3 an almost linear relationship is obtained (Fig. 7.3) with a correlation coefficient of -0.87.

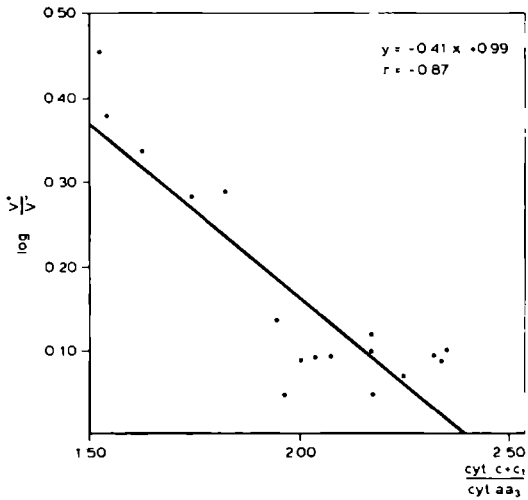

Fig. 7.3. Correlation between endogenous cytochrome c (expressed as the ratio between cyt. $c+c_1$ and cyt. aa_3) and the stimulation of pyruvate oxidation by exogenous cytochrome c.
V^+ and V^- represent the rate of pyruvate oxidation in the presence and absence of exogenous cytochrome c, respectively.

7.4. DISCUSSION

7.4.1. *Sucrose versus KCl isolation medium*

From the specific activities of cytochrome oxidase it can be concluded that the use of isotonic KCl medium during isolation of skeletal muscle mitochondria leads to mitochondrial suspensions which are more contaminated with protein from non-mitochondrial origin as compared with suspensions obtained after isolation in sucrose, which is in accordance with electron microscopic

findings. This explains the lower values found for the cytochrome content of isotonic KCl isolated mitochondria as compared with sucrose isolated mitochondria, since these values are expressed on protein base (Table 7.2). The rate of pyruvate oxidation is lower in isotonic KCl isolated mitochondria as compared with isotonic sucrose isolated mitochondria (Table 7.4). However, when these values are compared on base of spectrally measured cytochrome aa_3 (reduced with succinate plus KCN) or on base of cytochrome oxidase activity, the difference almost disappears. This implies that by taking protein as a reference base misleading results may be obtained. This problem is not encountered by using cytochrome oxidase as a reference base, which is probably due to the exclusively mitochondrial localization of this enzyme (12). Although the rate of pyruvate oxidation, when correctly expressed, is almost the same with either isotonic sucrose or isotonic KCl isolated mitochondria, mitochondria isolated in isotonic sucrose are superior to mitochondria isolated in isotonic KCl with respect to morphologic appearance (Fig. 7.1), specific activity of cytochrome oxidase (Table 7.1), respiratory control and P/O ratio (Table 7.3).

7.4.2. *Stimulation of pyruvate oxidation by cytochrome c*

It has been known for a long time that cytochrome c stimulates substrate oxidations in heart (13) and skeletal muscle (14) mitochondria. In a recent report (6) a stimulation of pyruvate oxidation by exogenous cytochrome c was reported varying between 34 % and 66 % depending on the type of muscle. In our experiments, pyruvate oxidation by skeletal muscle mitochondria isolated in isotonic sucrose or KCl shows a stimulation of about 20 % by exogenous cytochrome c. It seems therefore that the isolation medium employed by Van Handel et al. (6), which contained 175 mM KCl and 2 mM EDTA, resulted in a greater loss of cytochrome c than the isotonic sucrose or KCl media we used. Isolation in hypotonic sucrose results in such a loss of cytochrome c from the mitochondria (Table 7.2) that stimulation of pyruvate oxidation by exogenous cytochrome c increases to 100 %. The correlation between the ratio of the pyruvate oxidation rates in the presence and absence of exogenous cytochrome c and the ratio between cytochrome $c+c_1$ and cytochrome aa_3 (Fig. 7.3) indicates that the stimulatory effect of cytochrome c is due to an increased substrate concentration of cytochrome oxidase. So, the oxidation capacity of skeletal muscle mitochondria is limited by the amount of cytochrome c present after isolation. No stimulation by exogenous cytochrome c will be obtained at a

ratio between cytochrome $c+c_1$ and aa_3 of 2.4 (Fig. 7.3).
Although pyruvate oxidation is stimulated by addition of cytochrome c, no effect is seen on the P/O ratio (Table 7.3) which is in agreement with the results found by other investigators (4, 13, 14).

7.4.3. *Implication for clinical biochemistry*
Biochemical investigations with mitochondria isolated from muscle biopsy specimens are important for the diagnosis of mitochondrial myopathies (15, 16). The results reported in this chapter, although obtained with rat skeletal muscle mitochondria, have their consequences for workers in the clinical field. Especially the finding of discrepancies between pyruvate oxidation rates obtained with mitochondria isolated in different media which are merely related to the reference base used, stresses the importance of a good parameter for expressing measured activities.
Protein appeared to be not suitable. Good reference bases seem to be cytochrome oxidase activity or cytochrome aa_3, spectrophotometrically measured after reduction with succinate plus KCN.
Some investigators use incubation media containing cytochrome c when measuring the oxidative capacity of skeletal muscle mitochondria (18, 19). The routine addition of cytochrome c to these media seems not advisable when measuring the oxidative capacity of patients skeletal muscle mitochondria for two reasons. First, deficiencies of cytochrome aa_3 and cytochrome b have been reported (chapter 2). Therefore, cytochrome c deficiencies might exist as well and can be masked by addition of cytochrome c to the incubation medium, unless cytochromes are also measured separately. Secondly, cytochrome c can fullfill the role of terminal electron acceptor in case of inhibition of cytochrome oxidase (Fig. 7.2) and therefore mask a partial deficiency of cytochrome aa_3.

7.5. SUMMARY

Rat skeletal muscle mitochondria were isolated in three different isolation media: isotonic sucrose medium, isotonic KCl medium and hypotonic sucrose medium. The use of isotonic KCl medium during isolation resulted in a mitochondrial preparation which was more contaminated with protein from non-mitochondrial origin than the use of sucrose media. This could be concluded from electron microscopy and the specific activity of cytochrome oxidase. Furthermore, respiratory control index values and cytochrome content were lower in

these mitochondria. The ratio between cytochrome $c+c_1$ and cytochrome aa_3 was similar after isolation in both isotonic media but was markedly lower after isolation in hypotonic sucrose. The highest rate of pyruvate oxidation was obtained with isotonic sucrose isolated mitochondria when this rate was expressed on protein base. When expressed on base of spectrally measured cytochrome aa_3, reduced with succinate plus KCN, or on base of cytochrome oxidase activity, the rate of pyruvate oxidation was the same in isotonic sucrose- and isotonic KCl-isolated mitochondria.

Exogenous cytochrome c stimulated pyruvate oxidation about 20 % in mitochondria isolated in isotonic sucrose or KCl and about 100 % in mitochondria isolated in hypotonic sucrose. In the latter case, oxidation rates in the presence of exogenous cytochrome c approached the values found with mitochondria isolated in isotonic sucrose. A clear inverse relationship between the content of endogenous cytochrome $c+c_1$ and the stimulatory effect of exogenous cytochrome c is established.

Implications of these results for clinical studies of oxidative capacity of skeletal muscle mitochondria are discussed.

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CHAPTER 8

THE USEFULNESS OF MUSCLE HOMOGENATES AND MITOCHONDRIA IN THE STUDY OF MITOCHONDRIAL MYOPATHIES

8.1. INTRODUCTION

In the previous chapters the methods for the measurement of pyruvate dehydrogenase and citric acid cycle activity in intact muscle mitochondria as well as for the determination of the cytochrome content have been described. In this chapter the application of these methods for the study of mitochondrial myopathies will be illustrated.

Measurement of pyruvate dehydrogenase and citric acid cycle activity is not limited to mitochondrial suspensions but can also be performed in a muscle homogenate. The use of a homogenate has the advantage that less biopsy material is needed. It is our experience that isolation of a sufficient amount of mitochondria is only possible from biopsy specimens weighing at least 0.7 g. Isolation of mitochondria is necessary for the determination of RCI and P/O ratio and for measurement of cytochromes. RCI and P/O ratio cannot be measured in homogenates because of the high ATPase activity in the latter, resulting in increased state 4 oxidation rates. The concentration of mitochondrial pigments in a homogenate is too low to permit registration of a cytochrome spectrum. Based on these considerations the following sequence of determinations for the study of mitochondrial metabolism seems to be the most adequate one: a homogenate is prepared to measure pyruvate dehydrogenase and citric acid cycle activity and, when indicated, fatty acid metabolism. From the remaining part of the homogenate mitochondria can be isolated. If the yield of mitochondria is sufficient, RCI and P/O ratio as well as a cytochrome spectrum are measured. As an example of the application of these methods, the results obtained with a muscle biopsy specimen from a patient suspected to suffer from a mitochondrial myopathy will be presented.

8.2. BIOCHEMICAL STUDIES ON SKELETAL MUSCLE MITOCHONDRIA FROM A PATIENT

8.2.1. *Case report*

The patient, G.R., a boy, was born in 1963. His history was uneventful untill the summer of 1977 when he experienced a period of extreme fatigue provoked by

prolonged exercise. This period lasted about 5 months during which he was unable to perform any exercise. In this period he was admitted to a local clinic. General examination revealed no abnormalities, except for an extreme fatigue. Laboratory investigations, including CPK, were normal. EMG and ECG showed no abnormalities. Myasthenia was excluded. During hospitalization the fatigue decreased and the patient was dismissed in an improved condition in december 1977. In 1978 an ischemic forearm test as well as an exercise test were performed. Both revealed no abnormalities.
During hospitalization in 1977 a needle biopsy of the m.quadriceps was performed for histopathological examination. An excess of intra-myofibrillar lipid and glycogen was found (Fig. 8.1). Mitochondrial morphology was abnormal. The usual parallel arrangement of the cristae was lost and the cristae seemed rounded up. These results indicated the presence of a myopathy and suggested that this myopathy might be from mitochondrial origin. A surgical biopsy of the m.quadriceps was performed in 1978. Although the fatigue had disappeared at that time, electron microscopical investigations gave results which were similar to those found in the first biopsy specimen.

8.2.2. *Results and discussion*

Activities of pyruvate dehydrogenase, citric acid cycle and cytochrome oxidase were measured in homogenate (Table 8.1). These activities were all within the normal range. Studies with isolated mitochondria showed normal values for RCI and P/O ratio (Table 8.2) as well as a normal content of cytochromes (Table 8.3). Part of the mitochondrial pellet was used for electron microscopical examination. No abnormalities were noticed (Fig. 8.2). Since all investigations gave results which were similar to those found in controls, a deficiency of one of the enzymes involved in pyruvate metabolism seems unlikely.

8.3. GENERAL DISCUSSION

Incubations according to the scheme presented in Table 8.1 can be performed with 50 µl of a 10 % ^{w} /v homogenate for each assay. Since assays are performed in duplicate a total volume of 600 µl of a 10 % homogenate is needed, which corresponds to 60 mg wet weight of muscle tissue. Since some loss of material occurs during preparation of the homogenate and also some material is needed for determination of protein content and cytochrome oxidase activity, about 100 mg of muscle tissue is sufficient for the determinations shown in

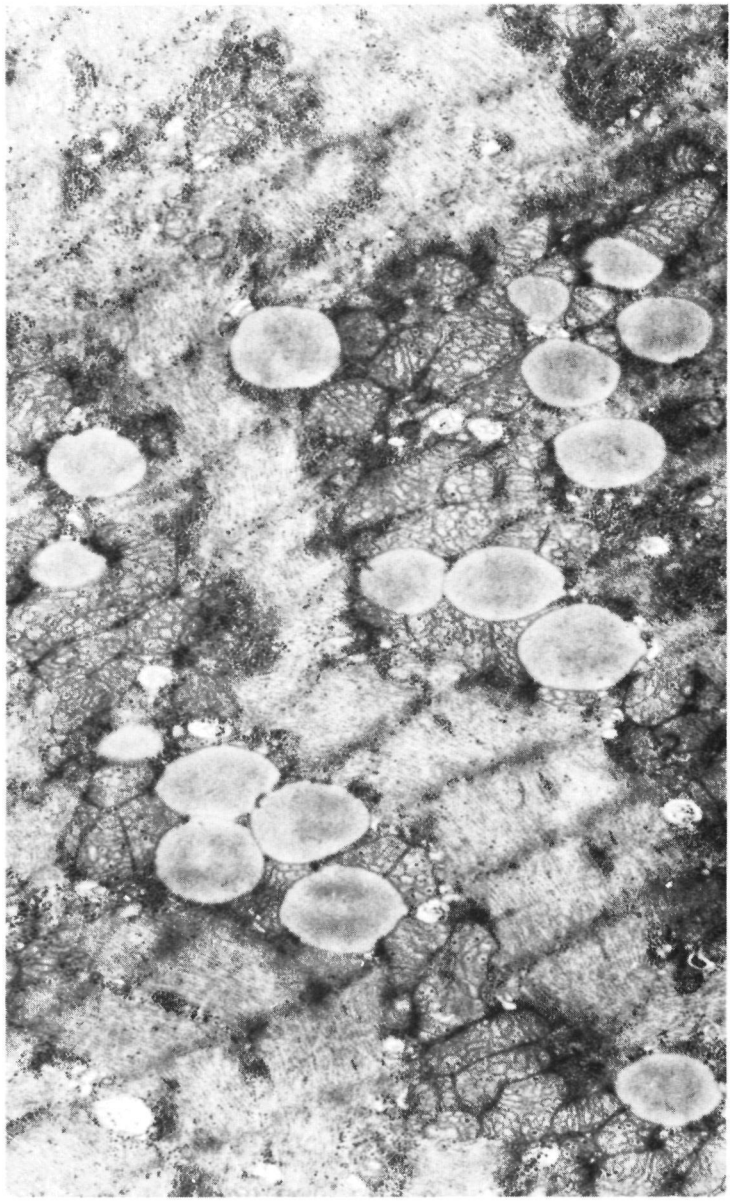

Fig. 8.1

Fig. 8.1. Electron micrograph of m.quadriceps from patient G.R. The tissue was fixed in glutaraldehyde and osmium tetraoxide, dehydrated in a graded series of alcohol and embedded in Epon 812. Ultrathin sections were stained with uranylacetate and leadcitrate. Note the abundance of lipid droplets inbetween the myofibrils and mitochondria. Also, the mitochondrial cristae show "rounding up". Magnification X 19.600.

TABLE 8.1.

$^{14}CO_2$ PRODUCTION FROM LABELED SUBSTRATES AND CYTOCHROME OXIDASE ACTIVITY IN MUSCLE HOMOGENATE FROM PATIENT G.R. AND CONTROLS

Incubation conditions were as described in the legends to Tables 3.3 and 4.1. The assays contained 0.15-0.30 mg homogenate protein. Values are expressed as nmol $^{14}CO_2$/mg protein.h.
Determination of cytochrome oxidase activity was performed at 37^{o} as described in 7.2. Values are expressed as nmol/mg protein.min.
Values of controls are the means $\pm$ S.D. of the number of experiments given in the parentheses.

	G.R.	controls	
[1-^{14}C]pyruvate + malate	256	324 $\pm$ 88	(7)
[1-^{14}C]pyruvate + carnitine	286	432 $\pm$ 106	(7)
[U-^{14}C]malate + pyruvate + malonate	400	376 $\pm$ 132	(7)
[U-^{14}C]malate + acetylcarnitine + malonate	330	432 $\pm$ 142	(7)
[U-^{14}C]malate + acetylcarnitine + arsenite	179	263 $\pm$ 93	(7)
[1-^{14}C]2-oxoglutarate	431	354 $\pm$ 117	(5)
Cytochrome oxidase	109	88 $\pm$ 33	(12)

TABLE 8.2.

OXYGEN CONSUMPTION, RCI AND P/O VALUES IN ISOLATED MUSCLE MITOCHONDRIA FROM PATIENT G.R. AND CONTROLS

Oxygen consumption was measured with 1 mM pyruvate and 1 mM malate as substrates at 37^{o} according to Max et al. (2). The rate of oxygen consumption is given in μgat O/mg protein.h.
Control values are the means $\pm$ S.D. of 4 experiments.

	Oxygen consumption		RCI	P/O
	state 3	state 4		
G.R.	26.7	3.6	7.4	2.9
Controls	23.0 $\pm$ 4.8	5.0 $\pm$ 1.4	4.6 $\pm$ 1.0	2.7 $\pm$ 0.2

TABLE 8.3.

CYTOCHROME CONTENT OF ISOLATED MUSCLE MITOCHONDRIA FROM PATIENT G.R. AND CONTROLS

Measurement of cytochromes was performed as described in 6.2. Reduction was achieved by addition of succinate plus KCN.
Values of controls are the means $\pm$ S.D. of 8 experiments.

	aa_3	b	$c+c_1$	aa_3	b	$c+c_1$
	(pmol/mg protein)			(relative amount)		
G.R.	443	327	646	1.0	0.74	1.46
Controls	374 $\pm$ 117	290 $\pm$ 71	648 $\pm$ 187	1.0	0.78	1.73

Fig. 8.2. Mitochondria isolated from a biopsy specimen (m.quadriceps) from patient G.R. For experimental details see 7.2. Magnification X 19.000.

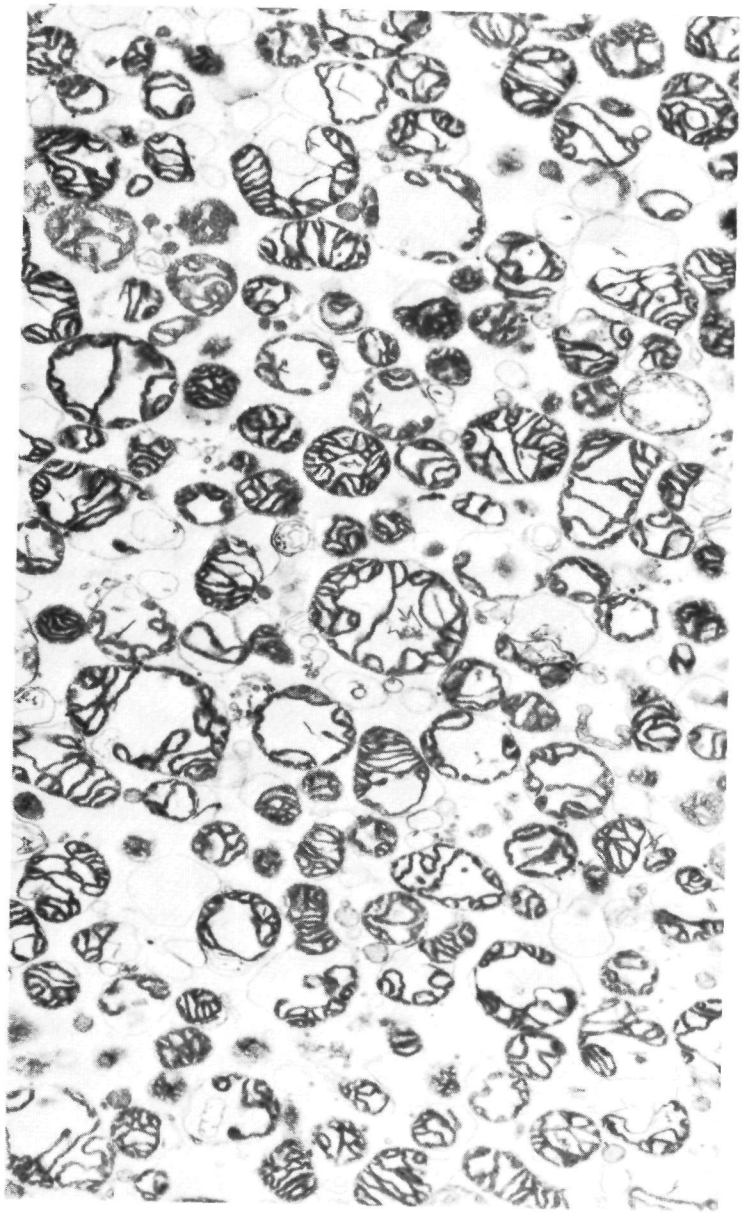

Fig. 8.2

Table 8.1. These determinations can also be performed in suspensions of isolated mitochondria but then an amount of 700 mg of muscle tissue is required for the isolation of mitochondria in normal conditions. Since the weight of a biopsy specimen is not always sufficient for isolation of mitochondria, we prefere to measure pyruvate dehydrogenase and citric acid cycle activity in muscle homogenates.

In addition to the patient reported above, biochemical investigations concerning mitochondrial metabolism were performed in a number of patients suffering from a myopathy which might be from mitochondrial origin. In none of these patients a defect in biochemical functioning of the mitochondria could be established. Therefore, the possibility must be considered that our methods are not always suitable to detect a defect in mitochondrial metabolism.

The procedure for the isolation of skeletal muscle mitochondria differs only in details from that employed by other investigators. All procedures are based on the disruption of muscle tissue, either mechanically (1,2) or enzymatically (3) or both (4,5), followed by centrifugation at low speed to remove cell debris and nuclei. The resulting homogenate is centrifuged at high speed to sediment the mitochondria. Since a number of defects were found in mitochondria isolated by such procedures (Chapter 2), it is likely to assume that abnormal mitochondria can be isolated as well in this manner. Further evidence for the latter is provided by the fact that with our isolation procedure and method for determination of the cytochrome content we were able to establish a deficiency of both cytochrome aa_3 and cytochrome b in a patient, who is described in detail elsewhere (6).

Ernster et al. (7) described an abnormal sedimentation behaviour of giant mitochondria isolated from a muscle biopsy specimen of a patient suffering from hypermetabolism. This might indicate inhomogeneity of the muscle mitochondrial population, at least under some pathological conditions. The biochemical defect, loose-coupling, was however present in both normally and abnormally looking mitochondria. To obtain information about the homogeneity of a mitochondrial population tissue fractionation studies can be performed by differential centrifugation. This procedure is time consuming and requires large amounts of tissue. Alternatively, skeletal muscle mitochondria can be separated into subpopulations by centrifugation on a density gradient (8,9). This procedure is subject to the same disadvantages as the afore mentioned procedure and, in addition, the mitochondria are sometimes exposed to extremely hypertonic conditions (8). The information obtained from our measurements

will be the resultant of both normally and abnormally functioning mitochondria, the former masking a possible defect present in the latter. However, there are no reasons to assume that this situation differs from the in vivo situation.

The measurements of the rates of substrate oxidation by the mitochondria were performed in the presence of optimal substrate concentrations. These concentrations will probably be far above those present in vivo and are therefore most suitable to detect a deficiency of a certain enzyme, whereas defects resulting in a decreased substrate affinity may remain unnoticed under these conditions. Furthermore, as outlined by Galton et al. (10), metabolic diseases may not only be caused by deficiencies of enzymes but also by defects in regulation of enzyme activities.

It is therefore our opinion that future biochemical research concerning skeletal muscle metabolism of patients should also be directed towards the development of methods which permit the study of mitochondrial metabolism under more physiological conditions. The system which seems most suited for this purpose is the isolated muscle cell, which has the advantage that the interrelationships between mitochondria and cytosol are preserved and that the mitochondria are in a more physiological environment.

8.4. REFERENCES

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A disturbance in functioning of skeletal muscle mitochondria may be one of the factors which can cause a myopathy. Such a myopathy is called a mitochondrial myopathy. The function of muscle mitochondria is to catalyze the oxidative catabolism of substrates coupled to the synthesis of energy. Pyruvate and fatty acids are the main substrates for skeletal muscle mitochondria, as outlined in Chapter 1. The experiments described in this thesis were performed to study the metabolism of mitochondria isolated from normal and diseased skeletal muscle. The investigations were limited to the oxidation of pyruvate and citric acid cycle intermediates.

In Chapter 2 a literature survey is presented concerning the mitochondrial myopathies described up to now in which a biochemical defect could be established.

Measurement of the rate of pyruvate oxidation in isolated muscle mitochondria can be performed by determining the rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate (Chapter 3). The stimulation of the rate of pyruvate oxidation by citric acid cycle intermediates and carnitine was studied and optimal conditions for measuring pyruvate oxidation in human muscle mitochondria were established.

Experiments concerning citric acid cycle activity during oxidation of pyruvate plus malate are described in Chapter 4. Oxidation of pyruvate is accompanied by utilization of malate, which is due to accumulation of 2-oxoglutarate and succinate. Reconstitution of the malate-aspartate shuttle in the incubation medium eliminated 2-oxoglutarate accumulation and resulted in accumulation of glutamate. Due to the accumulation of cycle intermediates it is not possible to measure overall flux through the citric acid cycle. The activities of the first and the second segment of the cycle can be determined by measuring the rate of $^{14}CO_2$ production from [U-^{14}C] malate and from [1-^{14}C] 2-oxoglutarate, respectively. The activities of the enzymes of the last segment of the cycle can be tested by determining the stimulation of [1-^{14}C]pyruvate oxidation with succinate or fumarate.

Oxidation of extra-mitochondrial NADH by isolated rat muscle mitochondria was studied by reconstitution of the malate-aspartate shuttle and the α-glycerophosphate shuttle (Chapter 5). The capacity for NADH oxidation by the reconstituted malate-aspartate shuttle was about 50 % higher than by the α-glycerophosphate shuttle. Considerable amounts of 2-oxoglutarate were withdrawn from the malate-aspartate shuttle by the 2-oxoglutarate dehydrogenase

complex and were oxidized to succinate. An unknown interaction between the reconstituted shuttles was found which enables the malate-aspartate shuttle to perform NADH transport in the presence of an uncoupler and the reconstituted α-glycerophosphate shuttle.

In Chapter 6 the measurement of cytochromes in mitochondria isolated from human muscle biopsy specimens is described. A reliable measurement of cytochromes after reduction with dithionite is not possible, due to contamination of suspensions of human muscle mitochondria with hemoglobin or myoglobin. Therefore, reduction should be performed with succinate plus KCN. Measurement of cytochromes is also possible in mitochondria isolated from muscle biopsy specimens stored at -70^{o}. However, storage of muscle tissue at -70^{o} leads to a preferential loss of cytochrome c from the mitochondria.

The effect of the use of different isolation media on some properties of rat muscle mitochondria is described in Chapter 7. Replacement of sucrose in the normal isolation medium by KCl resulted in mitochondrial preparations which were contaminated with protein from non-mitochondrial origin. Exposure of the mitochondria to a hypotonic shock during isolation resulted in a loss of cytochrome c from the mitochondria. The rate of pyruvate oxidation in isolated rat muscle mitochondria can be stimulated by addition of cytochrome c to the incubation medium. The increase in oxidation rate was dependent on the content of cytochrome c present in the mitochondria after isolation.

In Chapter 8 the results obtained with a muscle biopsy specimen from a patient suspected to suffer from a mitochondrial myopathy are presented. This Chapter ends with a discussion on the usefulness of muscle homogenates and mitochondria in the study of mitochondrial myopathies.

Een stoornis in het functioneren van skeletspier mitochondriën kan een van de factoren zijn welke een myopathie kunnen veroorzaken. Een dergelijke myopathie wordt een mitochondriale myopathie genoemd. De functie van spier mitochondriën is het katalyseren van het oxidatieve katabolisme van substraten gekoppeld aan de synthese van energie. Pyruvaat en vetzuren zijn de belangrijkste substraten voor skeletspier mitochondriën, zoals aangeduid in Hoofdstuk 1. De experimenten welke in dit proefschrift zijn beschreven werden uitgevoerd om het metabolisme van mitochondriën uit normale en pathologische skeletspier te bestuderen. Het onderzoek werd beperkt tot de oxidatie van pyruvaat en citroenzuur cyclus intermediairen.

In Hoofdstuk 2 wordt een literatuur overzicht gegeven van de tot nu toe beschreven mitochondriale myopathiën waarbij een biochemisch defect kon worden vastgesteld.

Het meten van de snelheid van pyruvaat oxidatie in geisoleerde spier mitochondriën kan uitgevoerd worden door de snelheid van de productie van $^{14}CO_2$ uit [1-^{14}C]pyruvaat te bepalen (Hoofdstuk 3). De stimulering van de snelheid van de oxidatie van pyruvaat door citroenzuur cyclus intermediairen en carnitine werd bestudeerd en er werden optimale condities vastgesteld voor het meten van de pyruvaat oxidatie in humane spier mitochondriën.

Experimenten betreffende de activiteit van de citroenzuur cyclus tijdens de oxidatie van pyruvaat plus malaat zijn beschreven in Hoofdstuk 4. De oxidatie van pyruvaat gaat gepaard met een verbruik van malaat, wat het gevolg is van de ophoping van 2-oxoglutaraat en succinaat. Reconstructie van de malaat-aspartaat shuttle in het incubatie medium elimineerde de ophoping van 2-oxoglutaraat en resulteerde in ophoping van glutamaat. Vanwege de ophoping van cyclus intermediairen is het niet mogelijk om de overall flux door de cyclus te meten. De activiteiten van het eerste en tweede segment van de cyclus kunnen bepaald worden door de snelheid van $^{14}CO_2$ productie te meten uit [U-^{14}C]malaat en [1-^{14}C]2-oxoglutaraat, respectievelijk. De activiteiten van de enzymen van het laatste segment kunnen bepaald worden door de stimulering van de oxidatie van [1-^{14}C]pyruvaat door succinaat of fumaraat te bepalen.

De oxidatie van extra-mitochondriaal NADH door geisoleerde rattespier mitochondriën werd bestudeerd door de malaat-aspartaat shuttle en de α-glycerofosfaat shuttle te reconstrueren (Hoofdstuk 5). De capaciteit voor NADH oxidatie via de gereconstrueerde malaat-aspartaat shuttle was ongeveer 50 % groter

dan via de α-glycerofosfaat shuttle. Aanzienlijke hoeveelheden 2-oxoglutaraat werden aan de malaat-aspartaat shuttle onttrokken door het 2-oxoglutaraat dehydrogenase complex en werden geoxideerd tot succinaat. Er werd een onbekende interactie tussen de gereconstrueerde shuttles gevonden welke de malaat-aspartaat shuttle in staat stelt om NADH te transporteren in aanwezigheid van een ontkoppelaar en de gereconstrueerde α-glycerofosfaat shuttle.
In Hoofdstuk 6 is de meting van cytochromen in mitochondriën geisoleerd uit humane biopten beschreven. Een betrouwbare meting van de cytochromen na reductie met dithioniet is niet mogelijk ten gevolge van de contaminatie van suspensies van humane skeletspier mitochondriën met hemoglobine of myoglobine. Daarom moet de reductie uitgevoerd worden met succinaat plus KCN. De meting van cytochromen is ook mogelijk in mitochondriën die geisoleerd zijn uit bij -70^{o} bewaarde biopten. Het bewaren van spierweefsel bij -70^{o} leidt echter tot een preferentieel verlies van cytochroom c van de mitochondriën.
Het effect van het gebruik van verschillende isolatie media op enige eigenschappen van rattespier mitochondriën wordt beschreven in Hoofdstuk 7. Vervanging van de sucrose in het normale isolatie medium door KCl resulteerde in mitochondriale preparaten die verontreinigd waren met eiwit van niet mitochondriale origine. Het blootstellen van de mitochondriën aan een hypotone shock tijdens de isolatie resulteerde in een verlies van cytochroom c van de mitochondriën. De snelheid van de pyruvaat oxidatie in geisoleerde rattespier mitochondriën kan gestimuleerd worden door toevoeging van cytochroom c aan het incubatie medium. De toename in oxidatie snelheid was afhankelijk van de hoeveelheid cytochroom c aanwezig in de mitochondriën na isolatie.
In Hoofdstuk 8 worden de resultaten vermeld die verkregen werden met een spierbiopt van een patient die verdacht werd aan een mitochondriale myopathie te lijden. Dit Hoofdstuk besluit met een discussie over de bruikbaarheid van spier homogenaten en mitochondriën voor het bestuderen van mitochondriale myopathiën.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 17 februari 1953 te Amsterdam. Hij doorliep de Rijks H.B.S. te Schagen alwaar in 1970 het diploma H.B.S.-B behaald werd. Hierna begon hij in 1970 zijn chemie studie aan de Universiteit van Amsterdam en behaalde in 1973 het kandidaatsexamen S_1. Het doctoraalexamen Scheikunde met als hoofdvak Biochemie (Prof.Dr. J.M. Tager), bijvak Klinische Chemie (Dr. H.J. v.d. Helm) en speciale richting Analytische Chemie (Prof.Dr. G. den Boef) werd in december 1975 behaald. Vanaf 1 januari 1976 was hij werkzaam als biochemicus op het laboratorium van de afdeling Paediatrie (Dr. P.J.J. van Munster) van het Sint Radboud Ziekenhuis te Nijmegen.

STELLINGEN

I

Voor de isolatie van skeletspier mitochondriën verdient het gebruik van een isotoon sucrose medium de voorkeur boven het gebruik van een isotoon KCl medium.

Dit proefschrift, hoofdstuk 7.

II

Het meten van een verschilspectrum van cytochromen na reductie met dithioniet kan aanleiding geven tot onbetrouwbare resultaten.

Dit proefschrift, hoofdstuk 6.

III

De door Blass et al. beschreven deficientie van het 2-oxoglutaraat dehydrogenase complex in gesonificeerde fibroblasten van patienten lijdende aan Friedreich's ataxie is moeilijk in overeenstemming te brengen met de normale waarden welke gevonden worden voor de oxidatiesnelheid van glutamaat in intacte fibroblasten.

Blass, J.P., Kark, R.A.P. and Menon, N.K. (1976) New Engl. J. Med. 295, 62 - 67.
Kark, R.A.P., Blass, J.P. and Engel, W.K. (1974) Neurology 24, 964 - 971.

IV

Bij het evalueren van resultaten welke verkregen zijn uit onderzoek van gekweekte adulte skeletspiercellen dient men zich rekenschap te geven van het feit dat in deze gekweekte cellen ook foetale kenmerken tot expressie komen.

DiMauro, S., Arnold, S., Miranda, A. and Rowland, L.P. (1978) Ann. Neurol. 3, 60 -66.
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V

Het "haastige spoed is zelden goed" is ook van toepassing op de pogingen om de duur van de analyse van aminozuren in fysiologische vloeistoffen zo kort mogelijk te maken.

VI

De stijging van de carnitine concentratie in de lever tijdens vasten kan verklaard worden door een afname in het gewicht van de lever bij een gelijkblijvende totale hoeveelheid carnitine.

Brass, E.P. and Hoppel, C.L.(1978) J. Biol. Chem. 253, 2688 - 2693.

VII

Het beoordelen van aanvragen voor subsidie voor wetenschappelijk onderzoek dient niet te geschieden door personen die zich in een competitie positie ten opzichte van de aanvrager bevinden.

VIII

Het verdient aanbeveling om tijdschriften in te binden op een manier die de mogelijkheid openlaat om later nog leesbare fotokopieen te maken.

IX

De verplichting tot het toevoegen van stellingen die geen betrekking hebben op het onderwerp van het proefschrift dient afgeschaft te worden.

Nijmegen, 22 december 1978. H. Bookelman.