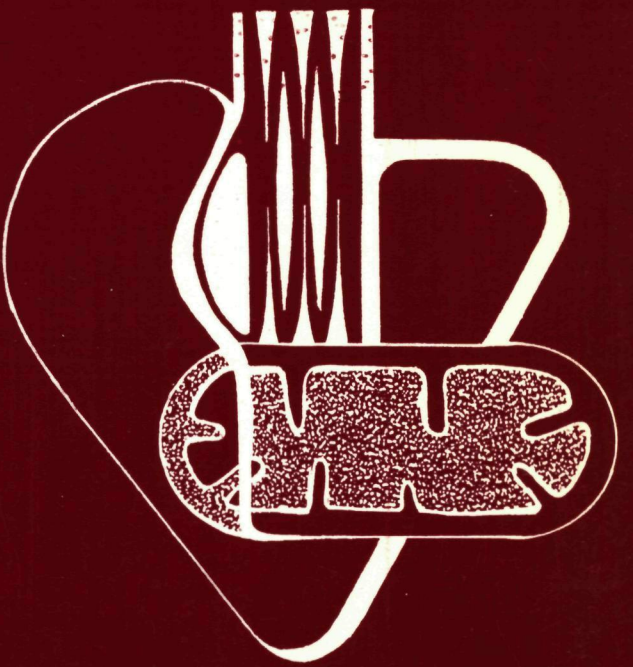
# FATTY ACID OXIDATION IN SKELETAL AND CARDIAC MUSCLE



J.F.C. GLATZ

# FATTY ACID OXIDATION IN SKELETAL AND CARDIAC MUSCLE

PROMOTORES:
PROF. DR. J.H. VEERKAMP
PROF. DR. S.L. BONTING

# FATTY ACID OXIDATION IN SKELETAL AND CARDIAC MUSCLE

## PROEFSCHRIFT

ter verkrijging van de graad van doctor in de
wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen
op gezag van de Rector Magnificus
Prof Dr J H G I Giesbers
volgens besluit van het College van Dekanen
in het openbaar te verdedigen
op vrijdag 9 december 1983
des namiddags te 2 uur precies

door

**JOHANNES FREDERICUS CAROLUS GLATZ**

geboren te Oss

1983

Drukkerij Aadé, Oss III

Aan mijn moeder en wijlen mijn vader
Aan Marie-Adele

*A man will turn over half a library*
*to make one book.*

*S. Johnson, 1824*

VI

Degenen die de afgelopen vier jaren bij de tot stand koming van dit proefschrift betrokken zijn geweest wil ik op deze plaats voor hun medewerking hartelijk danken. Zonder anderen daarbij te kort te willen doen, wil ik enkelen van hen met name vermelden.

De medewerkers van onze werkgroep dank ik voor de bijzonder prettige samenwerking, de gezellige werksfeer en hun persoonlijke steun.

Herman van Moerkerk stond altijd klaar voor het oplossen van technische en praktische problemen (ook op niet-wetenschappelijk terrein) en leverde een belangrijke bijdrage aan de interpretatie van meetgegevens. Zijn morele en artistieke steun zijn ook zeer gewaardeerd.

Veel dank komt toe aan Alga Jacobs, Camiel Baerwaldt en Anke Janssen, die met veel inzet en enthousiasme in het kader van hun doctoraalstudie een bijdrage aan het onderzoek hebben geleverd. René Megens en Ruth Knops hebben aan het onderzoek deelgenomen tijdens hun H.B.O.-B stage. Dorine Swinkels dank ik voor haar aandeel in de experimenten van hoofdstuk 3. Maria van Meijel (Laboratorium voor Immunochemie, Afdeling Kindergeneeskunde) verzorgde de immunochemische bepalingen beschreven in hoofdstuk 10.

De medewerkers van het Centraal Dierenlaboratorium, met name A.Busser en H. Eikholt, wil ik bedanken voor hun goede samenwerking. Bijzondere vermelding verdient hun inzet bij het op het gewenste tijdstip verkrijgen van nesten jonge ratjes.

De medewerkers van de Afdeling Thorax-hart-vaat Chirurgie van het Sint Radboudziekenhuis (hoofd Dr. S.H. Skotnicki) ben ik erkentelijk voor het beschikbaar stellen van humaan hartspier materiaal.

Met Dr. Herman-Jan Kempen (Gaubius Instituut, Gezondheidsorganisatie TNO, Leiden) is prettig samengewerkt bij een gedeelte van het onderzoek van hoofdstuk 10.

De altijd enthousiaste wijze waarop Dr. Peter Wirtz (Laboratorium Cyto- en Histologie) behulpzaam was bij het vergaren van morfologische kennis van de spier heb ik zeer op prijs gesteld.

I would like to thank Drs. R.K. Ockner (San Francisco, CA), K.M. Baldwin (Irvine, CA), M.E. Dempsey (Minneapolis, MN), M.M. Bhargava (Bronx, NY) and F.J. Pearce (Rochester, NY) for their hospitality and stimulating discussions on my visit to their laboratories.

De belangstelling en veelzijdige steun van mijn familie en vrienden en in het bijzonder van Marie-Adele waren tijdens de afgelopen periode onontbeerlijk.

Illustratie omslag: Theo Schetters

The investigations described in this thesis have been carried out at the Department of Biochemistry, Faculty of Medicine, University of Nijmegen, Nijmegen, The Netherlands, under the direction of Prof. Dr. J.H. Veerkamp.

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| CK | creatine (phospho)kinase |
| CoA | coenzyme A |
| DEAE | diethylaminoethyl |
| EC | enzyme commission number |
| EDTA | ethylene diaminetetraacetate |
| FABP | fatty acid-binding protein |
| FAD | flavine adenine dinucleotide |
| FFA | free fatty acids |
| $K_d$ | dissociation constant |
| $K_m$ | Michaelis-Menten constant |
| $M_r$ | relative molecular mass |
| $NAD^+$ | oxidized nicotinamide-adenine dinucleotide |
| SCP | squalene and sterol carrier protein |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SET | sucrose-EDTA-Tris buffer |
| TG | triacylglycerols |
| VLDL | very low density lipoproteins |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1. INTRODUCTORY REMARKS

Muscle tissue is responsible for locomotion and is important for the posture of man and animals. The cells of this highly specialized tissue are elongated in the direction of contraction, and therefore called muscle fibers. There are two general categories of muscle tissue in the body, cross-striated and smooth (nonstriated) muscle (Fig. 1.1). The latter forms, among others, the visceral and blood vessel musculature and is innervated by the autonomic nervous system, so that its contraction is not subject to voluntary control. Smooth muscle will not be considered in this study.

Upon microscopic examination, striated muscle exhibits regularly

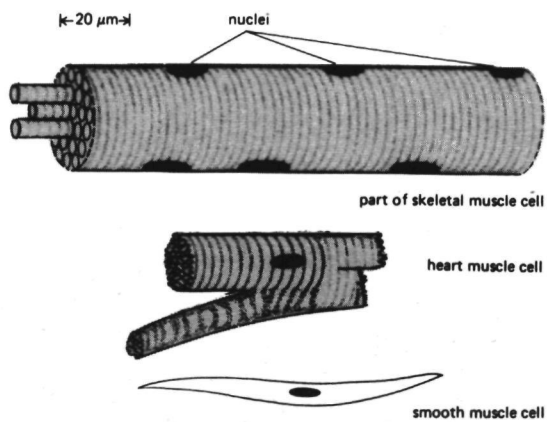



Fig. 1.1. Schematic presentation of the various kinds of muscle cells. Myofibrils are shown on the left side of the skeletal muscle cell. The sculptured surfaces on both ends of the ramified heart muscle cell represent the interdigitated junctions (intercalated discs) with neighbouring muscle cells, bearing junctional complexes that provide for mutual attachment and electrical conduction. (Reproduced, with permission, from Alberts *et al.*, 1983).

spaced transverse bands along the length of the fiber, and is subdivided into two distinct types, cardiac and skeletal muscle. Cardiac muscle makes up the wall of the heart and its rhythmical contraction is involuntary. On the contrary, contraction of skeletal muscle is voluntary. In most mammals, including man, the proportion of the total body weight represented by skeletal muscle varies with the age of the individual, being about 25% at birth and over 40% at maturity. In man it may decline to 30% or less in old age.

Large differences exist in the energy metabolism of different organs, e.g. liver, muscle, brain and kidney, but also among the various types of muscle. Skeletal muscle shows a lower metabolic capacity per kg tissue than liver and kidney, but its relatively large mass (18 and 11 times that of liver for man and rat, respectively) makes that this tissue plays a significant role in the overall metabolism of the body. Our knowledge about the regulation of energy metabolism of muscle, especially that of man, is limited. Extension of this knowledge will provide a better insight in the functioning of normal and pathological muscle and may form a basis for diagnosis and possibly therapy of metabolic disorders.

Previously, studies were performed at this laboratory focussed on fatty acid oxidation by skeletal and cardiac muscle of man and rat (see Thesis Van Hinsbergh, 1979). In those studies isolated mitochondria and a postnuclear fraction were applied to limit the complexity of the system. On this basis we further investigated the utilization of fatty acids for muscular energy production, using various cell-free and cellular systems. In the following sections the general properties of striated muscle tissue will be described and a survey will be given of the present knowledge on energy production and utilization in skeletal and cardiac muscle. A detailed description is given of the pathway of fatty acid oxidation. Since we also investigated the role of fatty acid-binding protein in the intracellular fatty acid metabolism, the current knowledge about this protein is reviewed. The chapter will be concluded with the aim and scope of this study.

## 1.2. MORPHOLOGY AND GENERAL PROPERTIES OF SKELETAL AND CARDIAC MUSCLE

Both skeletal and cardiac muscle are built up of large numbers of parallel orientated muscle fibers, enclosed in a thin envelope of connective tissue. For skeletal muscle, this connective tissue thickens towards

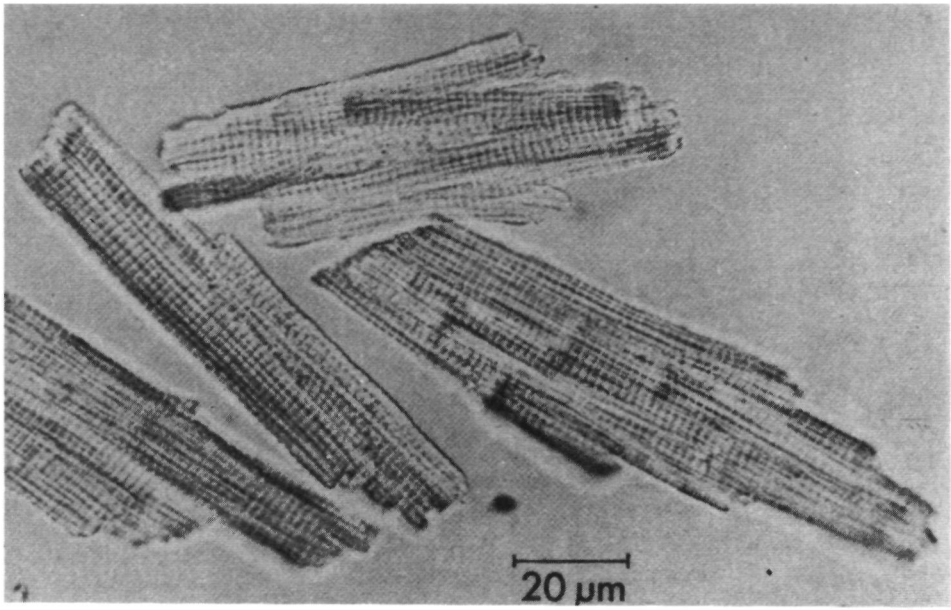



Fig. 1.2. Typical appearance of individual isolated rat myocardial cells under the light microscope. As in the intact tissue, the isolated cells branch freely and are irregular in cross section. Separation of the cells from its neighbours has occurred by cleavage at the intercalated disc.(Reproduced, with permission, from Dow *et al.*, 1981a).

the end of the muscle, thus forming the tendons by which the muscle is generally attached to the skeleton. Each individual muscle fiber is surrounded by a rich bed of capillaries, supplying substrates and oxygen and removing products and carbon dioxide.

Skeletal muscle fibers are multinucleated cells, formed by fusion of arrays of myoblasts during embryogenesis. The nuclei usually lie subsarcolemmally (Fig. 1.1). The cells are very long (up to 10-20 cm in man), in some muscles (e.g. m.soleus) they may run from the origin of the muscle to its insertion. The diameter of the adult mammalian muscle fibers ranges from 10 to 100 µm or more. Cardiac muscle cells are much shorter, have an irregular shape and are often branched (Figs. 1.1 and 1.2). The points of adhesion between adjacent cells can be recognized as transverse dark lines called intercalated discs. There are only a few nuclei in each cardiac cell,

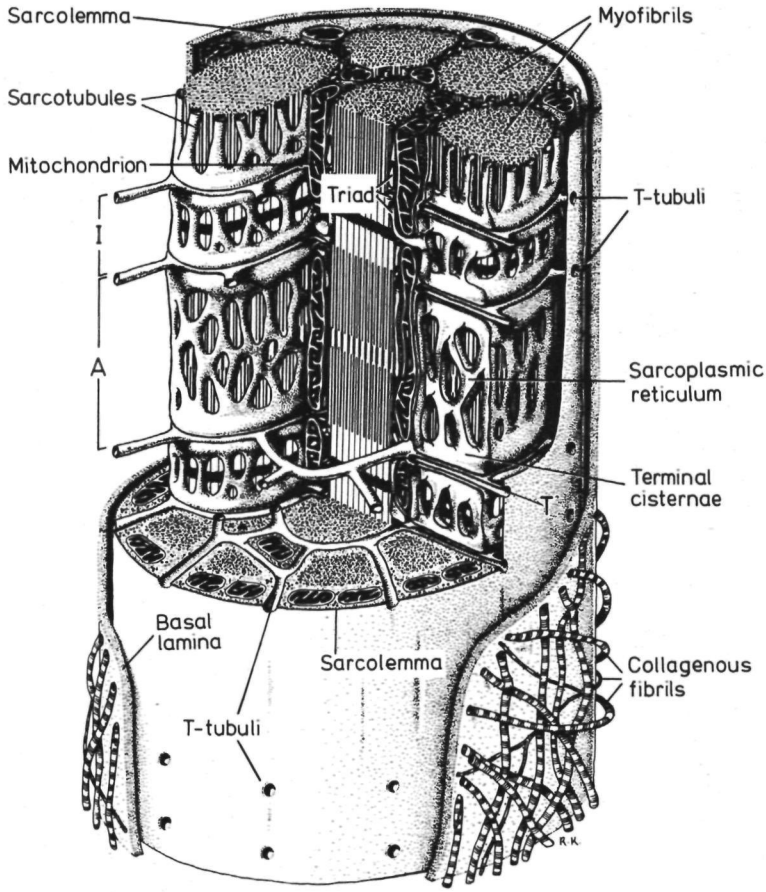



Fig. 1.3. Schematic presentation of part of a mammalian skeletal muscle fiber. The sarcoplasmic reticulum, which consists of longitudinal sarcotubules and transverse terminal cisternae, is in contact with the transverse tubuli at the so-called triad. The myofibrils are orientated in the direction of the fiber. Magnification, 24,000 x. (Reproduced, with permission, from Krstić, 1978).

usually situated deep in the interior of the fiber.

The striated muscle cell is supplied with an excitable membrane, the sarcolemma, which contains many tubular invaginations known as the T-system (Figs. 1.3 and 1.4), and which is involved in the onset of contraction. Cardiac muscle contains more and much larger transverse tubuli (T tubuli) than skeletal muscle. Apparently, no point in a cardiac muscle fiber is at more than 2-3 μm distance from the extracellular space. In skeletal muscle this distance is 4-25 μm (Bloom & Fawcett, 1975). These channels provide impor-

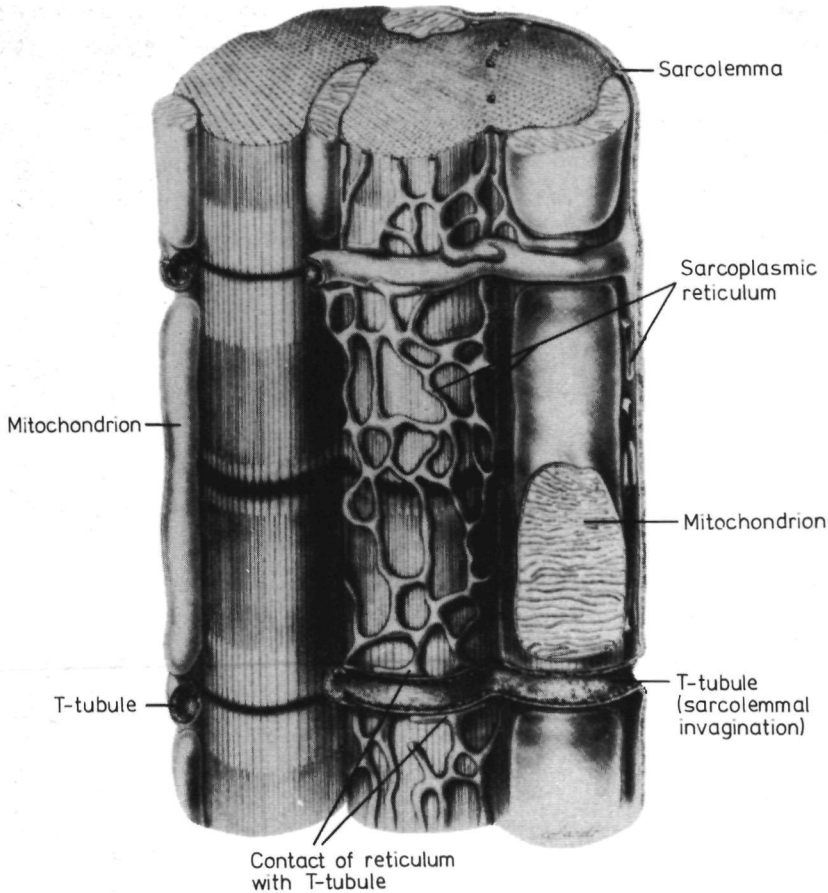



Fig. 1.4. Schematic presentation of part of a mammalian cardiac muscle cell. The mitochondria and T tubuli are more numerous and much larger than in skeletal muscle fibers. The relatively simple sarcoplasmic reticulum has few terminal cisternae and there are no triads. Instead, small terminal extensions of the reticulum are in contact with the cell membrane, either at the cell surface or at the T tubuli. (Reproduced, with permission, from Bloom & Fawcett, 1975).

tant additional surface for the exchange of metabolites between the muscle fiber and the extracellular space, especially in heart.

The sarcoplasm is nearly completely filled with contractile material, which in skeletal muscle is visible in the light microscope as discrete myofibrils but in cardiac muscle shows a more continuous nature (Figs. 1.3 and 1.4). The contractile proteins in skeletal and cardiac muscle are organized in functional units (sarcomeres), that will, however, not be consi-

dered in detail here. The sarcoplasmic reticulum surrounding the myofibrils comprises of a continuous system of membranes. It forms a separate compartment in the muscle fiber in which $Ca^{2+}$ ions are stored during rest. The sarcoplasmic reticulum corresponds to the endoplasmic reticulum of other cell types, but in mature muscle fibers it contains only few associated ribosomes. Its spatial orientation in the fiber is strictly related to the repeating order of the sarcomeres (Fig. 1.3). At regular intervals the sarcoplasmic reticulum forms an elaborate system of transversely orientated channels called terminal cisternae, which are in close contact with the T tubuli. In cardiac muscle the sarcoplasmic reticulum and terminal cisternae are less developed (Fig. 1.4). Small terminal expansions of the reticular network are closely applied to the membrane of the T tubuli.

Mitochondria may be located in longitudinal rows between the contractile material but also subsarcolemmally. Mitochondrial accumulations are often present at the nuclear poles and at sites where capillaries are situated just outside the muscle fiber. The number, size and distribution of mitochondria vary considerably in different types of skeletal muscle and in heart. Mitochondria of mammalian skeletal muscle usually are 1-2 μm long, but those in cardiac muscle are about 2.5 μm and may occasionally reach 7 or 8 μm (Bloom & Fawcett, 1975). Spherical lipid droplets and glycogen granules are often located near mitochondria.

Upon transmittance of a nerve pulse to the muscle, a wave of depolarization spreads over the sarcolemma and is conducted through the T-system to the sarcoplasmic reticulum. This causes a rapid efflux of $Ca^{2+}$ ions and eventually results in muscular contraction. The energy needed for this contraction and for the subsequent pumping back of the $Ca^{2+}$ ions into the sarcoplasmic reticulum is delivered by the hydrolysis of ATP.

In adult striated muscle three types of muscle fibers can be discerned, which show differences in contractile and metabolic properties, consistent with the functional demands of the muscle (Peter *et al.*, 1972). The classification commonly used for animal skeletal muscles is given in Table 1.1. It should be stressed that each muscle is composed of a mixture of the various fiber types. Oxidative (red) muscle fibers are usually found in the deep part of a muscle group, whereas glycolytic (white) fibers are superficially located, like in rat m.quadriceps femoralis (Baldwin *et al.*, 1972). Rat diaphragm contains about 60% slow-twitch oxidative fibers (Close, 1972). Adult rat m.soleus is almost exclusively (80-96%) built up of fast-twitch

*Table 1.1. Characteristics of animal skeletal muscle fiber types.*

| | Slow-twitch oxidative (SO) | Fast-twitch oxidative glycolytic (FOG) | Fast-twitch glycolytic (FG) |
|---|---|---|---|
| Color | red | intermediate | white |
| Mitochondrial content | moderate/high | high | low |
| Distribution of mitochondria | mainly intermyofibrillar | intermyofibrillar/subsarcolemmal | intermyofibrillar |
| Myoglobin content | high | high | low |
| Capillary supply | high | high | low |
| Fiber diameter | small | moderate | large |

Data were obtained from Baldwin *et al.*(1972), Close (1972) and Peter *et al.* (1972).

oxidative glycolytic fibers (Baldwin *et al.*, 1972; Close, 1972). In the rat the characteristic differences between the fibers are not yet apparent at birth, as all skeletal muscles then are slowly contracting. During subsequent postnatal development they increase their speed of contraction along different courses, which is the result of a process of differentiation of the fibers. The muscles acquire distinct patterns of fiber types in order to meet work demands (Close, 1972; Kelly & Rubinstein, 1980; Stockdale *et al.*, 1981).

## 1.3. MUSCLE METABOLISM

In muscle energy is needed for the performance of mechanical work, that is contraction and relaxation (reuptake of $Ca^{2+}$ ions into the sarcoplasmic reticulum), and restoration of the membrane potential after a nerve pulse, but also for other vital processes, including the maintenance of cellular organization, heat production during rest and anabolic activities. The energy for most of these processes is obtained from the hydrolysis of ATP. In resting mammalian muscle the ATP pool is only about 6 μmol/g and during contraction would be depleted in a few seconds, unless it is reple-

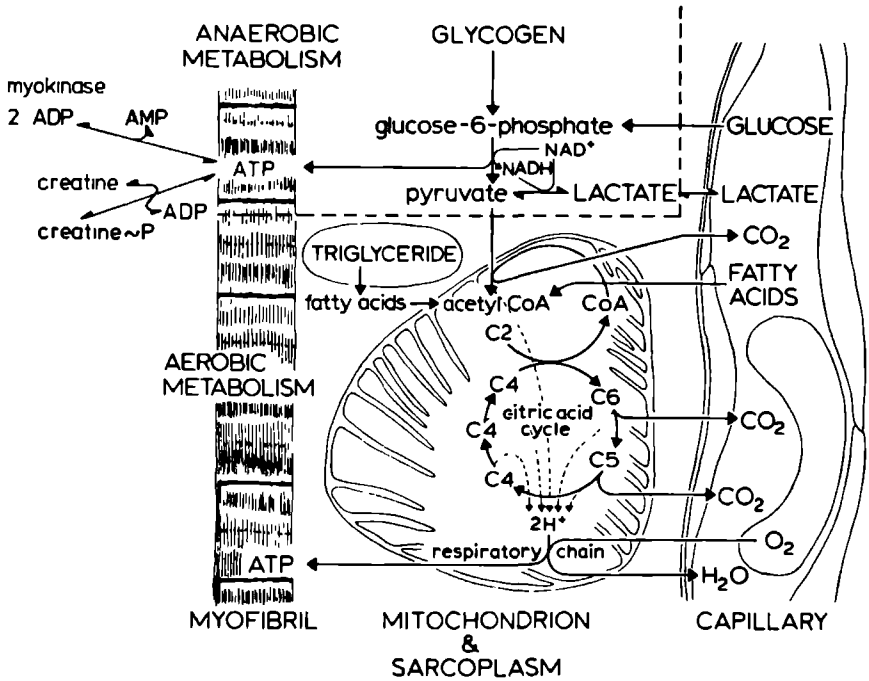



Fig. 1.5. Schematic presentation of energy metabolism in muscle. ATP can be regenerated by a one-step reaction from creatine phosphate or from ADP, and by multistep mechanisms from intracellularly stored substrates (glycogen and triacylglycerols) or from blood-borne substrates (glucose and fatty acids).

nished. Regeneration of ATP from ADP can occur in various ways: instantaneously from creatine phosphate, by anaerobic glycolysis (production of lactate), and by oxidative phosphorylation in mitochondria (aerobic pathway). When increasing amounts of ATP are utilized, these three processes contribute to its replenishment in this order.

*Creatine phosphate*

In muscle, the reserve of high energy phosphate is mainly stored in creatine phosphate (about 20-30 μmol/g in resting mammalian skeletal muscle). Creatine phosphate serves as a small, but instantaneously available source of energy. Rephosphorylation of ADP occurs by a single enzymatic step, catalyzed by creatine (phospho)kinase (Fig. 1.5). The formed creatine is rephosphorylated during rest or prolonged or intermittent exercise. Creatine ki-

nase exists in different iso-enzymes and with variable localization (Scholte, 1973a). In rat heart 33% of the total cellular creatine kinase activity resides in the myofibrils, 19% in the mitochondria, and 48% in the cytosol. For rat skeletal muscle these values are 4, 3 and 93%, respectively (Scholte, 1973a).

The role of creatine and creatine phosphate in muscle is not yet fully understood. The creatine kinase reaction has been considered to function in buffering the cellular ATP supply, thereby smoothing the metabolic response to changes in work load and providing a reservoir of ATP for brief periods of anoxia. Studies of Saks *et al.*(1978, 1980) and Kupriyanov *et al.*(1981) have shown that in rat heart the mitochondrial iso-enzyme, which is bound to the outer surface of the inner membrane, efficiently couples creatine phosphate synthesis to ATP production by its specific localization and coupling to adenine nucleotide translocase. On the basis of these observations it was proposed that the primary function of the system is to provide for energy shuttle from the mitochondria to the site of energy utilization (Wilson *et al.*, 1981; Seraydarian, 1982).

Another mechanism, by which the muscle ATP pool can be instantaneously replenished, involves transphosphorylation of two molecules of ADP by myokinase, but this reaction only occurs under extreme or pathological conditions.

*Anaerobic glycolysis and oxidative phosphorylation*

Ultimately, all energy required for ATP synthesis is obtained from the degradation of carbon-hydrogen bonds of various intramuscularly stored or blood-borne substrates: carbohydrates, fatty acids and amino acids. In the absence of oxygen ADP can be phosphorylated during glycogenolysis and glycolysis, in combination with lactate production (Fig. 1.5). Anaerobic glyco(geno)lysis generates, however, only a limited amount of ATP. Therefore, this pathway appears to be important only during a short period of muscle work and at a maximal load (Table 1.2). Oxidative degradation of substrates, occurring inside the mitochondria, delivers much more energy. Electrons are transferred from the substrates via $NAD^+$ and/or flavoproteins to molecular oxygen. The proton gradient generated over the mitochondrial inner membrane during the flux of the electrons through the respiratory chain, drives the synthesis of ATP and other processes, such as translocation of cations (Mitchell, 1977). The function of muscle mitochondria has recently been reviewed (Veerkamp, 1981).

*Contribution of substrates to muscular energy production*

The relative contribution of the various substrates to the muscular energy production is influenced by nutritional and hormonal conditions, the duration, intensity and kind of exercise (Table 1.2), training, muscle fiber composition, pathology, and also by the developmental stage of the individual. The main fuels for oxidative catabolism in muscle mitochondria are pyruvate (originating from glycogen, glucose, alanine or lactate) and fatty acids. The interaction of carbohydrate and fatty acid metabolism in skeletal and cardiac muscle in man was recently reviewed by Kaijser (1982). Since the intramuscular pools of triacylglycerols and glycogen are rather limited, skeletal and, especially, cardiac muscle mainly rely upon exogenous substrates for energy production. The contribution of glucose is increased after feeding and decreased during fasting in man (Wahren, 1979). In the post-absorptive state the net contribution of glucose is low, since a marked portion of the small amount of glucose taken up is returned to venous blood in the form of lactate, pyruvate and alanine (Wahren, 1979). In this condition fatty acids are the main fuel for the resting muscle. In conditions of sustained exercise or long-term fasting, branched-chain amino

*Table 1.2. Contribution of substrates to muscular energy production in man in the post-absorptive state.*

| Substrate | Recovery or rest | Exercise | | | |
|---|---|---|---|---|---|
| | | Short intense | Intermittent intense | Continuous moderate | |
| | | 1 min | 1 h | 1 h | 4 h |
| Creatine phosphate | - | ++ | (+) | - | - |
| Glycogen | - | ++ | (+) | + | - |
| Glucose | - | - | + | ++ | + |
| Fatty acids | + | - | ++ | ++ | ++ |
| Branched-chain amino acids | - | - | - | - | + |
| Acetoacetate | - | - | - | - | + |
| Lactate | + | - | (+) | - | - |

- no or little; + important; ++ marked; (+) alternatively utilization and production.

acids (Davies *et al.*, 1980; Rennie *et al.*, 1981) and acetoacetate (Berger *et al.*, 1978) are also used (Table 1.2).

Insufficient oxygen supply during the first minutes of exercise may cause a delay in energy production from oxidative phosphorylation and a high rate of anaerobic glycolysis (Hermansen, 1979). The supply of fatty acids to the muscle rises during sustained exercise by an increased blood flow and an increased lipolysis of triacylglycerols in the adipose tissue (Gollnick, 1977; Jones *et al.*, 1980). The increased supply to muscle leads to a higher rate of fatty acid oxidation and accumulation of citrate and/or acetyl-CoA in the muscle fiber (Rennie & Holloszy, 1977; Kaijser, 1982). These metabolites may inhibit phosphofructokinase or pyruvate dehydrogenase (Newsholme *et al.*, 1977; Kaijser, 1982) and in this way decrease glucose and glycogen utilization. In skeletal muscle fatty acids may contribute from 20 to 80% of the energy production (Fritz *et al.*, 1958; Bylund *et al.*, 1975; Hagenfeldt, 1979). The continuously working cardiac muscle is adapted to aerobic metabolism and preferentially oxidizes fatty acids, but may also use ketone bodies during fasting (Neely & Morgan, 1974). In the heart the relative contributions of carbohydrates and fatty acids to the substrate utilization are hardly altered by the work intensity of the heart, but more directly determined by the availability (i.e. plasma concentration) of the substrates (Kaijser, 1982).

## 1.4 FATTY ACID OXIDATION

### *Sources of fatty acids*

In blood plasma fatty acids are present non-covalently bound to albumin (free fatty acids) and esterified in triacylglycerols, forming part of lipoproteins. The free fatty acid (FFA) is the principal form utilized by muscle tissue. They arise mainly from lipolysis of triacylglycerols in adipose tissue. The half life of the plasma FFA is only 1-2 min (Spector & Fletcher, 1978). The plasma concentration of FFA usually varies from 0.2 to 0.6 mM, but may rise up to 1.5 mM during prolonged fasting or exercise, during recovery after exercise, and during disease (Gollnick, 1979; Spector & Fletcher, 1978; Hagenfeldt, 1979; Jones *et al.*, 1980). The albumin concentration is about 0.6 mM in both man and rat, so that the FFA:albumin molar ratio ranges from 0.3 : 1 to 2.5 : 1. The albumin-bound fatty acids comprise the long-chain fatty acids found in adipose tissue, i.e. (both man and rat)

mainly palmitate (25-30 mol%) and oleate (30-40%), and also palmitoleate (4-6%), stearate (8-10%), linoleate (13-17%) and arachidonate (3-5%) (Grimmer *et al.*, 1963; Hagenfeldt, 1968; Rogiers, 1981). The various fatty acids are bound by albumin with different affinity (Spector, 1975; Kragh-Hansen, 1981). Albumin has a few high-affinity binding sites for fatty acids and a greater number of weaker sites. The many other compounds that are bound by albumin, such as bilirubin, steroids and drugs (e.g. clofibric acid), may influence the fatty acid binding by the protein.

Fatty acids taken up in muscle also originate from chylomicron and VLD-lipoprotein triacylglycerols, which are hydrolyzed by lipoprotein lipase localized at the endothelium in the muscle capillaries (Fig. 1.6). In skeletal muscle the lipoprotein lipase activity is highest in muscles with a high content of oxidative muscle fibers (Borensztajn *et al.*, 1975; Linder *et al.*, 1976; Mackie *et al.*, 1980). In human and rat skeletal muscle and in rat heart the activity of this enzyme increases during starvation (Linder *et al.*, 1976; Lithell *et al.*, 1978; Mackie *et al.*, 1980) and prolonged exercise (Borensztajn *et al.*, 1975; Lithell *et al.*, 1979; Mackie *et al.*, 1980; Oscai *et al.*, 1982). Exercise increases also the utilization of intramuscularly stored triacylglycerols, mainly during the initial phase of moderate exercise, when the blood supply of FFA is not yet elevated (Essén, 1978), and during prolonged vigorous exercise (Reitman *et al.*, 1973; Essén, 1977; Stankiewicz-Choroszucha & Górski, 1978; Oscai *et al.*, 1982).

The reported triacylglycerol contents of rat heart and skeletal muscle vary from 1 to 25 μmol/g wet weight tissue (Masoro, 1967; Reitman *et al.*, 1973; Abumrad *et al.*, 1978; Stankiewicz-Choroszucha & Górski, 1978; Stearns *et al.*, 1979; Okano *et al.*, 1980; Stam & Hülsmann, 1981; Oscai *et al.*, 1982). This large variation is due to differences of age and strain of the animals and of the diet, but also of the conditions for sampling and measurement, since breakdown of triacylglycerols is rapid after decapitation (Frayn & Maycock, 1980). Generally, the triacylglycerol content is in red muscle fibers > intermediate muscle fibers ≈ cardiac muscle > white muscle fibers (Reitman *et al.*, 1973; Okano *et al.*, 1980). Training of rats decreases the muscle triacylglycerol stores, but increases the capacity to use them (Oscai *et al.*, 1982). Lipoprotein lipase may play a role in regulating the concentration of endogenous triacylglycerols in rat heart and skeletal muscle (Palmer *et al.*, 1981; Oscai *et al.*, 1982).

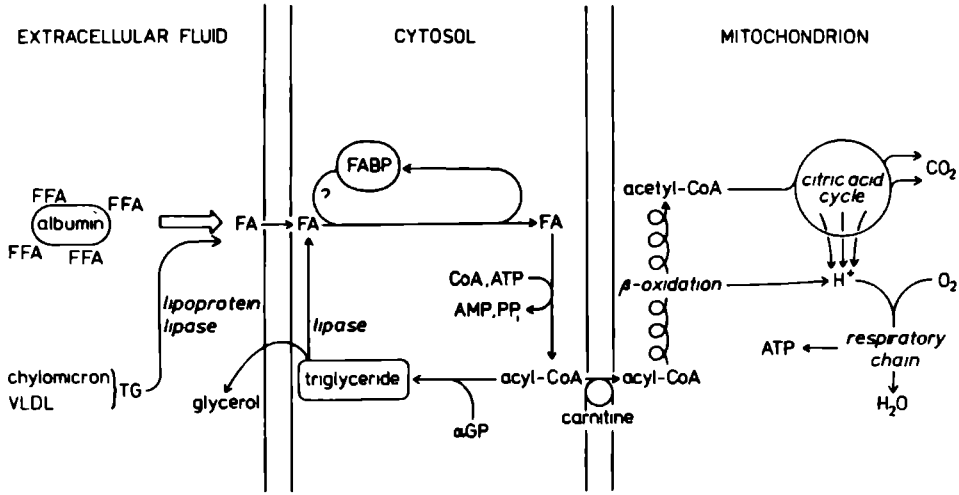



Fig. 1.6. Route and conversion of long-chain fatty acids from blood-borne substrate to degradation products $CO_2$ and $H_2O$. FFA, free fatty acid; TG, triacylglycerols; FABP, fatty acid-binding protein; VLDL, very low density lipoproteins; αGP, α-glycerophosphate.

*Uptake and cellular transport of fatty acids*

The rate of fatty acid uptake and utilization by peripheral tissues depends to a large extent on the concentration of fatty acids in the blood, but also on the metabolic activity of the tissue in question. The initial rate of uptake of FFA by human and rat skeletal and cardiac muscle is linearly related to their plasma concentration, both at rest and during exercise (Spector *et al.*, 1965; Hagenfeldt, 1979; Kaijser, 1982). Uptake of FFA appears to be an energy-independent process (Spector, 1971). An increase of the FFA:albumin molar ratio increases the unbound fatty acid concentration (Spector, 1975), and also the myocardial uptake of FFA (Oram *et al.*, 1973; Miller *et al.*, 1976; Hagenfeldt, 1979). When the rate of removal of intracellular FFA is increased by increasing the rate of oxidative metabolism, the rate of FFA uptake by isolated rat hearts also increased (Neely *et al.*, 1976). Thus, the net rate of FFA uptake by cardiac muscle depends on the concentration gradient across the muscle cell membrane (Idell-Wenger & Neely, 1978; Van der Vusse *et al.*, 1983). Under normal conditions the concentration of unbound FFA in plasma will vary from 1 to 10 μM (Spector, 1975; Idell-Wenger & Neely, 1978). With the exception of arachidonate, no

differences in the fractional extraction of individual FFA by human muscle were observed (Hagenfeldt, 1975), so that palmitate and oleate appear the main FFA taken up into muscular tissue. Whether such factors as pH, lactate and binding properties of circulating and cellular proteins influence the uptake of the various individual FFA differently is not known.

On their way from the blood plasma to the interior of the cell, the fatty acids have to pass several barriers, i.e. the capillary endothelium, the interstitial space, and the muscle cell membrane. The present knowledge about the mechanisms underlying the transport across these barriers is still rather limited. In contrast to liver and kidney, the muscle capillary wall does not contain pores through which substances could diffuse freely. Electron microscopic studies, however, revealed the presence of vesicles in the cytosol of the capillary endothelial cell (Bloom & Fawcett, 1975). These vesicles may be the main route for the transcapillary movement of molecules. Transport of fatty acids and other lipophilic compounds was also suggested to occur by lateral movement in a continuous interface composed of the plasma and vesicle membranes of the endothelial cell, and possibly also the plasma membrane of the muscle cell (Scow *et al.*, 1980).

Transport of fatty acids in the interstitial space may also be mediated by albumin. Its presence in the interstitial space, and also in the transverse tubular system, has been demonstrated with immunocytochemical methods (Yokota, 1982). The concentration of albumin in interfiber fluid from guinea pig skeletal muscle is about 42% of that in the vascular compartment (i.e. about 0.24 mM) (Creese *et al.*, 1962). The concentration in human suction blister fluid, which might be regarded as representative of muscle interstitial fluid, was 29% of that of plasma (Vermeer *et al.*, 1979).

The mechanism by which the fatty acid gains entry to the tissue cell interior once separated from albumin is still a point of discussion. It was long assumed that the uptake of fatty acids occurs by simple diffusion through the plasma membrane. During the last years, however, several investigators have proposed the existence of a specific membrane carrier system for the transport of fatty acids. Stremmel *et al.*(1983a,b) presented evidence for the existence of an oleate binding protein in the rat liver cell plasma membrane. A bilirubin binding protein of $M_r$ 55,000 was earlier identified in this membrane (Reichen & Berk, 1979; Wolkoff & Chung, 1980). Fatty acid uptake by rat adipocytes is facilitated by a saturable, phloretin-inhibitable mechanism that is presumably protein in nature (Abumrad *et*

*al.*, 1981). Samuel *et al.* (1976) and Paris *et al.* (1978) also observed a saturable fatty acid uptake process in embryonic chick cardiac cells. On the other hand, De Grella & Light (1980a,b) concluded that fatty acids penetrate adult rat heart myocytes by simple diffusion and that the saturable uptake component observed in previous studies reflected predominantly metabolism. Several of these workers reported that at a high concentration of unbound fatty acids permeation occurs, however, by a non-saturable process, resembling passive diffusion (Samuel *et al.*, 1976; De Grella & Light, 1980a; Abumrad *et al.*, 1981). Therefore, the putative function of a membrane protein in the uptake and/or transfer of long-chain fatty acids remains to be established.

Weisiger *et al.* (1981, 1982) argued that in liver the single-pass extraction ratios of many albumin-bound ligands (e.g. long-chain fatty acids, bile acids, bilirubin, dyes and drugs) appear rather high compared to the *in vitro* observed rate of dissociation of these ligands from albumin. On the basis of kinetic studies of oleate and bilirubin uptake in relation to albumin concentration in the perfused rat liver and isolated rat hepatocytes, they proposed that the hepatic uptake of these compounds is not determined by the fraction of the free ligand, but involves saturable interaction of the albumin-ligand complex with a specific receptor site on the liver cell surface (Weisiger *et al.*, 1980, 1981, 1982; Ockner *et al.*, 1983). Evidence for a specific albumin receptor on the rat adipocyte surface was also reported (Brandes *et al.*, 1982). Several other investigators could, however, not confirm the existence of the postulated receptor in liver (Stremmel *et al.*, 1983a; Stollman *et al.*, 1983), adipose tissue (Abumrad *et al.*, 1981) and cultured human leukemic myeloid cells (Morand *et al.*, 1982). Stremmel *et al.* (1983a) suggested that the above-mentioned apparent discrepancy is explained by the fact that albumin-ligand complexes dissociate more rapidly *in vivo* than predicted from studies *in vitro*.

The intracellular transport of the FFA is assumed to be mediated by low-$M_r$ fatty acid-binding proteins (FABP), which are found in the cytosol of many tissues, including skeletal and cardiac muscle (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). It is not yet known whether FABPs are also involved in or influence the uptake of FFA by the muscle, e.g. by promoting FFA desorption from the plasma membrane. These proteins will be discussed in section 1.5.

*Fatty acid oxidation pathway*

Long-chain fatty acids are mostly degraded by β-oxidation, although other pathways also are known. α-Oxidation, the removal of one carbon atom at a time from the carboxyl end of the molecule, has been detected in brain tissue (Fulco, 1967). Cytoplasmic ω-oxidation was found in liver and may contribute to total fatty acid oxidation in states of excessive fat catabolism(starvation, diabetes) (Bjorkhem, 1978; Kam *et al.*, 1978; Gregersen & Ingerslev, 1979). The presence of α- and ω-oxidation has not been established in skeletal muscle and heart.

The β-oxidation of long-chain fatty acids was traditionally envisaged to occur solely in mitochondria. It is now well established, however, that peroxisomes also contain enzymes for fatty acid degradation (Lazarow & De Duve, 1976; Masters & Holmes, 1977, 1979; De Duve, 1983). Peroxisomes (or microbodies) are spherical, single-membrane organelles of about 0.5 - 1 μm, which were named after their involvement in the production (oxidases) and degradation (catalase) of hydrogen peroxide. Although cytochemical methods demonstrated the presence of peroxisomes in liver, striated muscles and other tissues (Hand, 1974;Christie & Stoward, 1979; Tolbert, 1981), peroxisomal fatty acid oxidation has mainly been studied in liver. There are only a few recent reports on peroxisomal fatty acid oxidation in heart (Small *et al.*, 1980; Norseth *et al.*, 1982, 1983) and skeletal muscle (Small *et al.*, 1980; Shumate & Chokski, 1981).

The first step in the cellular metabolism of fatty acids is the conversion of the relatively unreactive carboxylic group into the more reactive coenzyme A thioester. This process, referred to as activation, is catalyzed by long-chain acyl-CoA synthetases (Fig. 1.7), which in the liver are located on the outer mitochondrial membrane, the peroxisomal membrane and the endoplasmic reticulum. The energy for this reaction is obtained from hydrolysis of ATP, by which AMP is formed. In liver the $K_m$ for palmitate is similar (about 5 μM) for the mitochondrial and the peroxisomal synthetases (Krisans, 1980), indicating that at this level there is no preference for fatty acids to enter the mitochondrial or peroxisomal β-oxidation pathway. In heart the long-chain acyl-CoA synthetase activity appears, however, to be exclusively localized on the mitochondrial membrane (Normann *et al.*, 1983). The activation reaction is controlled by the acyl-CoA:coenzyme A ratio and the total concentration of coenzyme A (Idell-Wenger & Neely, 1978). The formed long-chain acyl-CoA is relatively insoluble in water and

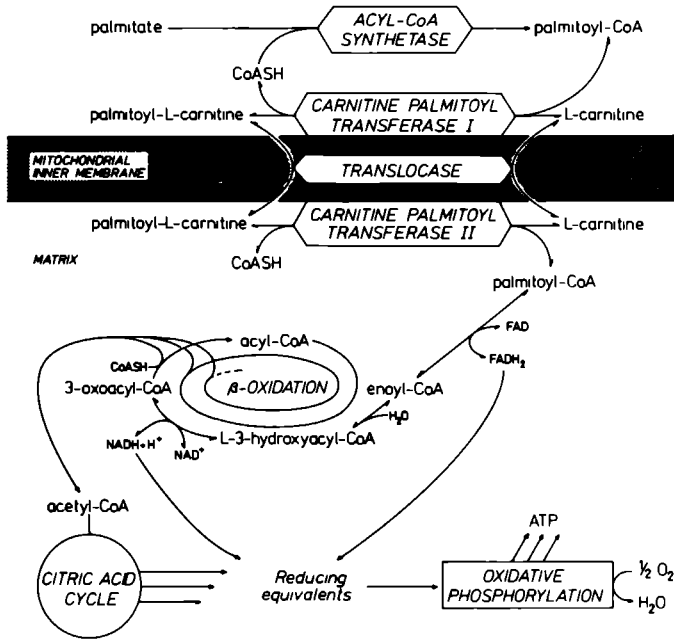



Fig. 1.7. Schematical presentation of the pathway of long-chain fatty acid oxidation in mitochondria. (Modified after Aprille, 1976).

like fatty acids is probably bound to FABP (Mishkin & Turcotte, 1974) and lipid membranes. Short-chain and medium-chain acyl-CoA synthetases are also present in heart and liver, but the former shows a very low activity in rat skeletal muscle (Scholte & Groot, 1975).

The acyl-CoA ester can be used either for the synthesis of phospholipids and triacylglycerols in the cytosol or for the oxidation within mitochondria or peroxisomes. In order to undergo mitochondrial oxidation, the acyl residues have to be transported into the mitochondrial matrix via a carnitine-dependent shuttle mechanism, which is composed of two carnitine acyltransferases, localized on the outer and inner surface of the mitochondrial inner membrane, respectively, and acylcarnitine translocase (Fig. 1.7) (Bremer, 1977; Hoppel, 1982). By this mechanism the cytosolic and mitochondrial pools of coenzyme A are strictly separated. The transport of acylcarnitine across the inner membrane involves a 1:1 exchange with L-carnitine, which means that the content of carnitine on both sites of the membrane

remains constant under physiological conditions. Exchange transport appears to be driven by concentration gradients and is independent of metabolic energy. The transfer of the acyl group from coenzyme A to L-carnitine is promoted by the high cytosolic carnitine:coenzyme A ratio, which is even higher in muscle than in liver (Oram *et al.*, 1975; Idell-Wenger & Neely, 1978). The physiological function of this ratio may be to direct fatty acids toward either lipid synthesis or oxidation. The relatively low rates of lipid synthesis in heart compared to liver are in agreement with this conception (Idell-Wenger & Neely, 1978). Based on substrate specificity mitochondria may contain four distinct carnitine acyltransferases, that are reactive towards short-chain acyl esters (chain length of 2-10 carbon atoms), medium-chain acyl esters (6-10 carbon atoms), long-chain acyl esters (8 carbon atoms or higher), and branched-chain acyl esters. The carnitine long-chain acyltransferase (carnitine palmitoyltransferase) is exclusively a mitochondrial enzyme in liver, heart and skeletal muscle (Hoppel, 1982), but the carnitine short-chain and carnitine medium-chain acyltransferases are also present in peroxisomal and microsomal fractions of

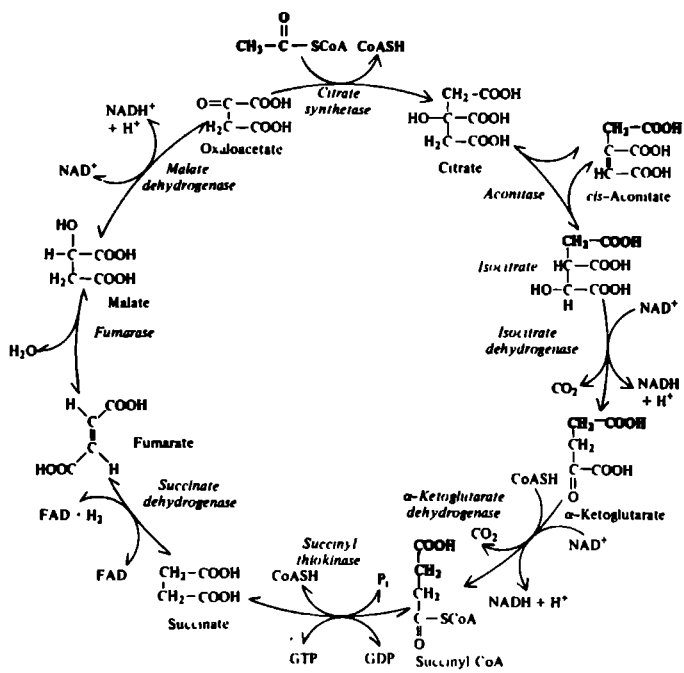



Fig. 1.8. Citric acid cycle.

rat liver (Markwell *et al.*, 1973, 1976; Bieber *et al.*, 1982). Beef heart mitochondria, however, do not contain a specific carnitine medium-chain acyltransferase (Clarke & Bieber, 1981).

Intramitochondrial degradation of the fatty acyl-CoA ester occurs via β-oxidation, whereby acetyl-CoA residues are split off from the carboxylic end of the fatty acid by a repeated sequence of processes: oxidation, hydration, oxidation and thiolytic cleavage (Fig. 1.7). The four tightly linked enzymes, which catalyze the reactions, are: fatty acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase (Engel, 1978; Middleton, 1978). Some of these enzymes are known to exist in various forms with different chain-length specificity. During β-oxidation one molecule of flavoprotein and one of $NAD^+$ are reduced and one molecule acetyl-CoA is formed per cycle. The major part of the energy present in the fatty acid molecule is released during the oxidation of the acetyl-CoA in the citric acid cycle (Fig. 1.8). The reducing equivalents (NADH and $FADH_2$) produced during β-oxidation and citric acid cycle are oxidized in the respiratory chain, located in the inner mitochondrial membrane, to lead to the generation of ATP via oxidative phosphorylation (Fig. 1.7). Proper functioning of the mitochondrial energy supply is dependent on the presence and adequate order of all the constituents of this chain and the ATP-synthesizing system.

Peroxisomes do not contain a carnitine-dependent transport system for long-chain acyl-CoA esters, since carnitine palmitoyltransferase activity is not found in these organelles and peroxisomal acyl-CoA oxidation is not stimulated by carnitine (Markwell *et al.*, 1977; Mannaerts *et al.*, 1979). Peroxisomal β-oxidation occurs similar to that of mitochondria, except that in the first reaction, which is catalyzed by a flavin-linked oxidase, two electrons are transferred from acyl-CoA to molecular oxygen, with the for-

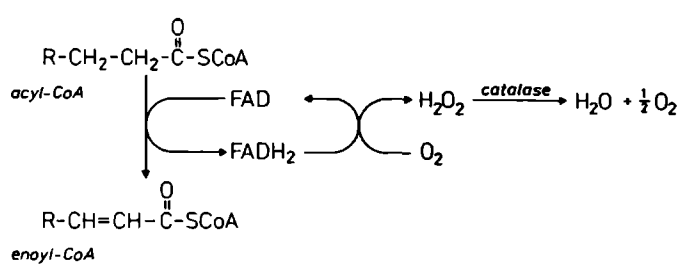



Fig. 1.9. Scheme of the first step of peroxisomal β-oxidation.

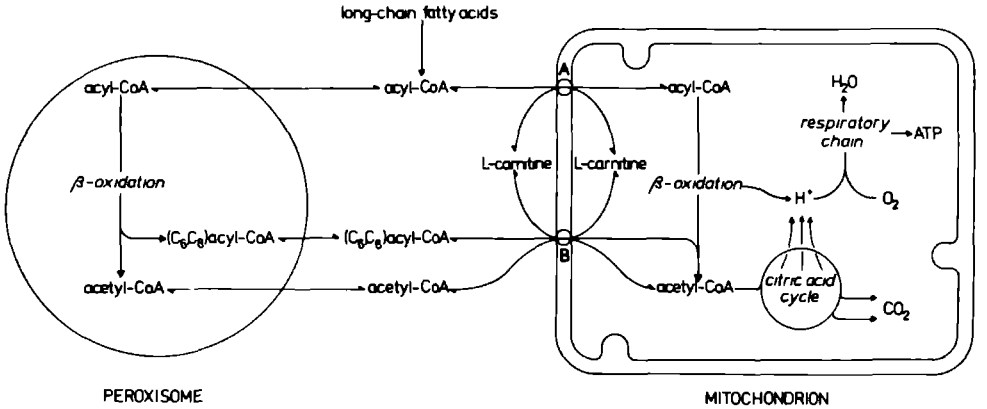



Fig. 1.10 Interaction of peroxisomal and mitochondrial long-chain fatty acid oxidation.

mation of enoyl-CoA and $H_2O_2$ (Fig. 1.9). The latter product is decomposed by catalase. The subsequent three reactions are the same as those in mitochondrial β-oxidation, but the enzymes all are different from their mitochondrial counterparts (Inestrosa *et al.*, 1980; Osumi *et al.*, 1980; Lazarow, 1981a). It is unknown at present, whether the NADH generated at the third β-oxidation reaction is reoxidized within the peroxisome or in the cytosol (Lazarow, 1982).

The peroxisomal β-oxidation system has a maximal activity towards acyl-CoA esters of 12-18 carbon atoms and little towards $C_6$ and $C_4$ acyl-CoA esters (Lazarow, 1978; Osumi & Hashimoto, 1978; Hryb & Hogg, 1979), so that the oxidation is not complete. At the most five β-oxidation cycles were observed with palmitoyl-CoA oxidation by isolated rat liver peroxisomes (Lazarow, 1978; Osmundsen *et al.*, 1979). Therefore, the acetyl-CoA and short-chain acyl-CoA esters that accumulate as the end products of peroxisomal β-oxidation may be transported to the mitochondrion for entry into the citric acid cycle or mitochondrial β-oxidation pathway, respectively (Fig. 1.10), or may be used for biosynthetic purposes elsewhere. Carnitine and/or FABP may play a role in the removal of these peroxisomal products. The peroxisomal β-oxidation reactions are insensitive to KCN when assayed *in vitro*, because they are not linked directly to the mitochondrial respiratory chain (Lazarow & De Duve, 1976; Mannaerts *et al.*, 1979).

In liver, the relative contribution of peroxisomes to the overall oxidation of palmitate and oleate appears to be only 5-10% under normal conditions (Mannaerts *et al.*, 1979; Thomas *et al.*, 1980). Peroxisomes may, however, play a significant role in the oxidation of very long chain fatty acids (22 carbon atoms or higher) (Neat *et al.*, 1981).

*Control of fatty acid oxidation*

In heart muscle, the main factors controlling the rate of fatty acid oxidation appear to be: (i) fatty acid supply by the blood, which is determined by the diet and hormonal control of fatty acid mobilization from liver and adipose tissue, (ii) the level of high-energy phosphates and the redox state of the mitochondria, and (iii) the availability of coenzyme A both in the mitochondrial compartment and in the cytosolic space (Oram *et al.*, 1973; Idell-Wenger & Neely, 1978). The transport system for acyl units across the inner mitochondrial membrane may function as an important regulatory site for fatty acid oxidation (Idell-Wenger, 1981; Idell-Wenger *et al.*, 1982). Malonyl-CoA, which inhibits the outer carnitine palmitoyltransferase in liver and heart (McGarry *et al.*, 1978a,b, 1983; Saggerson & Carpenter, 1981), and short-chain acyl-CoA esters may thereby play a regulatory role.

## 1.5. FATTY ACID-BINDING PROTEIN

*Introduction*

In 1972, Ockner *et al.* and Mishkin *et al.* reported that the cytosol of rat liver, intestinal mucosa, adipose tissue, kidney and of cardiac and skeletal muscle contains a protein of molecular weight of about 12,000, that *in vitro* exhibits a high affinity for the non-covalent binding of oleate. The protein appeared to be identical with the smaller of two previously described hepatic cytosolic anion-binding proteins, called proteins B and A by Ketterer *et al.* (1967) and Y (or ligandin) and Z by Levi *et al.* (1969). Therefore, three different designations of the protein are used: fatty acid-binding protein (FABP), Z-protein and protein A. Throughout this study we will, however, use the former term.

The presence of this binding protein in the cytosol was originally suggested in order to explain the observed differences in intestinal absorption among long-chain fatty acids and to account for the apparent faci-

lity with which these fatty acids traverse the cytosol, thus ascribing a role to the protein intracellularly much like that of albumin extracellularly (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). Subsequent research established that FABP does not only act to 'solubilize' long-chain fatty acids but also influences the activities of various enzymes of lipid metabolism. Since many other lipidic compounds are also bound by FABP, including long-chain acyl-CoA esters, bilirubin, bile acids and salts, aminoazo dyes, hexachlorophene, sulfobromophtalein, cholesterol and sex steroid hormones and their sulphates, it is clear that the protein may potentially play an important role in cellular lipid metabolism. In the following text we present a brief review of the current knowledge of FABP.

*Physicochemical characterization of FABP*

Initially FABP was identified by measuring the association of radioactively labeled fatty acids with specific protein fractions by gel-filtration (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). Comigration with labeled fatty acid or with colored organic anions, like bilirubin or sulfobromophtalein, is still commonly applied for the purification and assay of FABP. However, this technique appears not adequate, since fatty acids and organic anions also bind with a high affinity to most types of gel material (Mishkin *et al.*, 1975; Stein *et al.*, 1976) and therefore may dissociate from the proteins as filtration proceeds. Furthermore, due to this interaction and to competition of FABP with other proteins (e.g. albumin) for available ligand, the method appears not suitable for the quantitation of FABP in protein samples. The presence of labeled ligands on FABP also impairs characterization and further studies of the protein.

FABP has been purified and characterized from rat, rabbit and bovine liver, human and rat adipose tissue, rat and pig heart and rat intestinal mucosa. Purification is usually performed by gel-filtration on Sephadex G-75, anion- or cation-exchange chromatography, and sometimes preparative isoelectric focusing (e.g. Ketterer *et al.*, 1976; Fournier *et al.*, 1978; Barbour & Chan, 1979; Ul-Haq *et al.*, 1982). Other applied purification steps include ammonium sulphate precipitation (Matsushita *et al.*, 1977; Trulzsch & Arias, 1981; Takahashi *et al.*, 1982), heat treatment (Billheimer & Gaylor, 1980) and affinity chromatography (Ockner *et al.*, 1982). The molecular weight, isoelectric point and amino acid composition of the various purified preparations are summarized in Tables 1.3 and 1.4. Recently, Ul-Haq *et*

*Table 1.3. Properties of FABP and of some related proteins.*

| Origin | Designation | $M_r$ value | Isoelectric point(s) | Reference |
|---|---|---|---|---|
| Rat liver | FABP | 12,080[c] | 6.9 | Ockner *et al.*, 1982 |
| | FABP | 10,000[c] | 5.9 | Suzue & Marcel, 1975 |
| | Z | 12,500[c] | 5.2 | Trulzsch & Arias, 1981 |
| | | | 6.0 | |
| | | | 7.3 | |
| | Z | 14,300[a] | n.d. | Takahashi *et al.*, 1982 |
| | | 14,000[c] | | |
| | Z | 12,000[b] | 7.0 | Matsushita *et al.*, 1977 |
| | | 11,000[c] | | |
| | Z | n.d. | 1.6-2.6 | Rüstow *et al.*, 1982 |
| | | | 8.8-9.3 | |
| | A | 14,000[c] | 5.0 | Ketterer *et al.*, 1976 |
| | | | 5.9 | |
| | | | 7.6 | |
| | Band C | 11,450[a] | 5.5 | Billheimer & Gaylor, 1980 |
| | | 12,000[c] | 6.0 | |
| | | | 7.0 | |
| | $SCP_1$ | 15,000[a] | 7.0 | Dempsey *et al.*, 1981 |
| | | 16,000[b] | | |
| | | 16,200[c] | | |
| Rabbit liver | Z | 12,000[b] | 7.0 | Matsushita *et al.*, 1977 |
| | | 11,000[c] | | |
| Bovine liver | FABP | 12,000[b] | 5.9[d] | Haunerland & Spener, 1980 |
| Rat adipose tissue | FABP | 12,000[c] | n.d. | Ul-Haq *et al.*, 1982 |
| Human adipose tissue | FABP | 12,000[c] | n.d. | Ul-Haq *et al.*, 1982 |
| Rat intestinal mucosa | FABP | 12,100[b] | 5.6 | Ockner & Manning, 1974 |
| | | 12,400[c] | | |
| Rat heart | FABP | 12,000[c] | 5.0 | Fournier *et al.*, 1978 |
| Pig heart | FABP | 12,000[c] | 4.8 | Fournier *et al.*, 1983a |

[a] Amino acid analysis
[b] Gel filtration
[c] SDS-gel electrophoresis
[d] In the presence of oleate $pI = 5.0$
n.d., not determined

*al.* (1983) reported the purification of FABP from rat and monkey lung tissue but these authors did not yet give physicochemical data of the proteins.

All FABPs studied so far have a molecular weight of 11,000-14,000, but show large differences in isoelectric point (Table 1.3). Three groups reported that upon isoelectric focusing, homogeneous rat liver FABP precipitates at pH 5.0-5.5, 5.9-6.0 and 7.0-7.6 (Ketterer *et al.*, 1976; Billheimer & Gaylor, 1980; Trulzsch & Arias, 1981). The three forms of FABP had a closely similar amino acid composition and ultraviolet spectrum, were immunochemically identical, but exhibited different affinities for long-chain fatty acids and sulfobromophtalein, the form with the highest p*I* value having the highest affinity. Heterogeneity in FABP may, however, result from interaction of the protein with ampholytes or exogenous ligands (Trulzsch & Arias, 1981), or may be caused by denaturation (Dempsey *et al.*, 1981). Binding of long-chain fatty acids to bovine liver FABP (Haunerland & Spener, 1980) and human serum albumin (Basu *et al.*, 1978) was reported to be accompanied by a lower p*I* value. Therefore, it is likely that FABP from all tissues and species examined is a neutral protein with p*I* 7.0-7.5 (Table 1.3).

The amino acid composition has yet only been determined for rat liver FABP (Table 1.4). The data reported by the various investigators are in close agreement. The primary structure of rat liver FABP was recently reported (Takahashi *et al.*, 1982).

Tables 1.3 and 1.4 also give the physicochemical properties of Band C protein and of sterol carrier protein$_1$ ($SCP_1$) from rat liver. These proteins stimulate a number of microsomal enzyme activities involved in cholesterol biosynthesis from squalene (Billheimer & Gaylor, 1980; Dempsey *et al.*, 1981). These data, and the findings that $SCP_1$ shows a high affinity for long-chain fatty acids and organic anions (Ritter & Dempsey, 1973; Grabowski *et al.*, 1976; Dempsey *et al.*, 1981) and stimulates hepatic fatty acid activation and esterification by membrane-bound enzymes (Daum & Dempsey, 1979, 1980), indicate that Band C protein and $SCP_1$ are mutually identical and very similar or identical to FABP (cf. chapter 10).

Immunochemical assays for the detection and quantitation of FABP were developed for rat liver, adipose tissue and jejunal FABP (Ockner & Manning, 1974; Stein *et al.*, 1976; Ketterer *et al.*, 1976; Trulzsch & Arias, 1981; Rüstow *et al.*, 1982; Ockner *et al.*, 1982; Ul-Haq *et al.*, 1983). With the assays immunochemical cross-reactivity was observed for FABPs from various rat tissues (Ockner & Manning, 1974; Rüstow *et al.*, 1982 ) but not for human and

*Table 1.4. Amino acid composition of FABP and related proteins of rat liver (in residues/1000 amino acid residues).*

| Amino acid | FABP | Z[b] | Z[c] | A[d] | Band C | $SCP_1$ |
|---|---|---|---|---|---|---|
| | Ockner *et al.*, 1982 | Trulzsch & Arias, 1981 | Takahashi *et al.*, 1982 | Ketterer *et al.*, 1976 | Billheimer & Gaylor, 1980 | Dempsey *et al.*, 1981 |
| Asp | 87 | 78 | 86 | 97 | 99 | 89 |
| Thr | 94 | 81 | 91 | 101 | 112 | 94 |
| Ser | 48 | 33 | 47 | 52 | 53 | 36 |
| Glu | 131 | 142 | 139 | 152 | 139 | 136 |
| Pro | 16 | 12 | 12 | n.d.[e] | 21 | 16 |
| Gly | 96 | 102 | 93 | 94 | 100 | 102 |
| Ala | 17 | 17 | 18 | 19 | 20 | 16 |
| Cys[a] | 17 | 13 | 6 | n.d. | 7 | 10 |
| Val | 91 | 107 | 93 | 79 | 71 | 95 |
| Met | 53 | 21 | 54 | 50 | 25 | 54 |
| Ile | 66 | 70 | 70 | 60 | 66 | 67 |
| Leu | 48 | 53 | 49 | 51 | 57 | 51 |
| Tyr | 25 | 14 | 23 | 19 | 25 | 23 |
| Phe | 47 | 49 | 47 | 51 | 49 | 47 |
| His | 15 | 17 | 17 | 16 | 18 | 16 |
| Lys | 130 | 156 | 139 | 141 | 129 | 134 |
| Arg | 18 | 35 | 17 | 18 | 19 | 17 |
| Trp | 0 | n.d. | n.d. | 0 | n.d. | 0 |

[a] Determined as carboxymethyl cysteine
[b] Given for the protein of p*I* 7.2
[c] The second peak after DEAE-cellulose purification
[d] The protein of p*I* 7.6 (form III)
[e] n.d., not determined

rat FABP (Rüstow *et al.*, 1982; Ul-Haq *et al.*, 1983). The presence of immunochemically distinct FABPs in the rat tissues is, however, not excluded.

Analysis of the binding of long-chain fatty acids to protein is hampered by their low aqueous solubility and their low rate of diffusion through dialysis membranes, so that a complete Scatchard plot can usually not be obtained. With equilibrium-dialysis studies, Ketterer *et al.* (1976)

found that rat liver FABP shows a $K_d$ for palmitate of 1 μM and for palmitoyl-CoA of 0.14 μM. For sulfobromophtalein and several steroids they observed a class of strong binding sites ($K_d$ 0.1 - 3 μM) and several weaker sites (Ketterer *et al.*, 1976). On the basis of coelution of different amounts of radioactively labeled ligands with cytosolic proteins from a gel-filtration column, Mishkin *et al.* (1972) estimated the $K_d$ for oleate to be 2.8 μM and Mishkin & Turcotte (1974) found the $K_d$ for palmitoyl-CoA to be 0.24 μM. With this technique it was also found that the relative binding was greater for unsaturated than for saturated fatty acids (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). With the use of a charcoal binding assay, Warner & Neims (1975) observed an apparent $K_d$ for hexachlorophene and bilirubin of 20 and 50 μM, respectively.

Ockner *et al.* (1982) demonstrated that in rat liver 60% of the cytosolic long-chain fatty acids are associated with the FABP-containing fraction. 50-60% of these endogenous fatty acids are unsaturated (mainly oleate, linoleate and erucate). This is a substantial enrichment in these acids compared to plasma and indicates that FABP preferentially binds unsaturated fatty acids (Burnett *et al.*, 1979; Rüstow *et al.*, 1982; Ockner *et al.*, 1982). The saturated fatty acids are mainly palmitate (20-30%) and stearate (8-14%). The amount of endogenous fatty acids associated with rat liver FABP was reported to be 0.46 (Burnett *et al.*, 1979), 0.70 (Rüstow *et al.*, 1982) and 0.26 mol/mol FABP (Ockner *et al.*, 1982). Rüstow *et al.* (1982) also found that phospholipids (0.41 mol/mol FABP) and small amounts of mono-, di- and triacylglycerols and of cholesterol were endogenously bound by FABP. However, Burnett *et al.* (1979) and Ockner *et al.* (1982) found virtually no detectable phospholipid.

Delipidation of FABP obviously is important for the proper study of its binding characteristics and effects on metabolic events. However, only few investigators delipidated their preparations, mostly with 20% (v/v) n-butanol, by the method of Morton (1955), or with diisopropyl ether, by the method of Cham & Knowles (1976). Trulzsch & Arias (1981) reported that the delipidation procedure employing n-butanol was highly denaturing for hepatic FABP, but Fournier *et al.* (1978) and Barbour & Chan (1979) did not mention difficulties using this method. Using a Sephadex G-25 binding assay, Ockner *et al.* (1982) found that the fatty acid-binding affinity of liver FABP hardly changed or decreased after delipidation with diisopropyl ether. Hence, a suitable non-denaturing delipidation procedure is greatly needed (cf. chapter 8).

Rat liver cytosol was also found to contain a fatty acid-binding peptide of $M_r$ 1500-2000 and p*I* 5.7 (Suzue & Marcel, 1975; Rustow *et al.*, 1978). The peptide does not appear to be an artifactual product of FABP, but to be present within mitochondria and microsomes, and physiologically not in the cytosol (Rüstow *et al.*, 1979).

*Functional properties of FABP*

Although much information about the role of FABP in cellular long-chain fatty acid metabolism has come forth by indirect evidence, it has also been possible to directly attribute physiological properties to the protein. Purified rat heart FABP was demonstrated to be able to transfer its bound fatty acid to the mitochondrial β-oxidative system (Fournier *et al.*, 1978). Appelkvist & Dallner (1980) provided evidence for the involvement of FABP in peroxisomal fatty acid oxidation. By binding long-chain fatty acids and their acyl-CoA esters, rat liver FABP exercises indirect control on the activity of acetyl-CoA carboxylase (Lunzer *et al.*, 1977) and of several membrane-bound enzymes, such as mitochondrial ATP/ADP-translocase (Barbour & Chan, 1979), mitochondrial and microsomal acyl-CoA synthetase (Wu-Rideout *et al.*, 1976; Ockner & Manning, 1976; Burnett *et al.*, 1979), microsomal glycerol 3-phosphate acyltransferase and diacylglycerol acyltransferase (Mishkin & Turcotte, 1974; Burnett *et al.*, 1979), and microsomal methyl sterol oxidase, hydroxymethylglutaryl-CoA reductase and acyl-CoA: cholesterol acyltransferase (Grinstead *et al.*, 1983). Purified FABP also stimulates hepatic triacylglycerol synthesis in cell-free systems (O'Doherty & Kuksis, 1975; Burnett *et al.*, 1979; Iritani *et al.*, 1980).

In several studies significant correlations were observed between the tissue content of FABP and the rate of fatty acid uptake or utilization in several conditions involving dietary, hormonal and pharmacological manipulations. By means of a quantitative radial immunodiffusion assay for purified intestinal FABP, Ockner & Manning (1974) found that the FABP concentration in mucosa from the proximal and middle part of the rat jejuno-ileum exceeded that in the distal third, and furthermore that FABP concentrations were responsive to changes in the fat content of the diet. With immunochemical techniques, Rustow *et al.* (1982) demonstrated that increasing the fat content of the diet from 1 to 25% also significantly rises the cytosolic concentration of FABP in rat liver, adipose tissue and heart. The liver cytosol of obese Zucker rats shows a 1.7-fold higher fatty acid-binding ac-

tivity per mg protein when compared to that of lean rats (Morrow *et al.*, 1979).

The utilization of oleate and its incorporation into triacylglycerols were greater in hepatocyte suspensions from adult female rats than from males, and correspond to a higher FABP concentration in liver cytosol of female rats (Ockner *et al.*, 1979). Furthermore, these sex differences in both fatty acid utilization and FABP content could be reproduced by hormone administration to castrated rats (Ockner *et al.*, 1980). Clofibrate feeding to rats increases the hepatic content of FABP as well as the uptake rate of free fatty acids (Fleischner *et al.*, 1975; Renaud *et al.*, 1978; Kawashima *et al.*, 1982), and the tissue content of long-chain acyl-CoA, acetyl-CoA and total coenzyme A (Ball *et al.*, 1979). The decreased tissue concentration of long-chain acyl-CoA in several Morris hepatomas with respect to control and host liver is accompanied by a markedly lower cytosolic FABP concentration (Mishkin & Halperin, 1977; Mishkin *et al.*, 1977).

*Concluding remarks*

FABP may play an important role in cellular lipid metabolism, as can be concluded from the observations that FABP binds a wide variety of ligands, influences the activity of several membrane-bound enzymes involved in lipid metabolism, and that its cytosolic concentration is relatively high (4-5% of cytosolic protein in rat liver, Ockner *et al.*, 1982) and correlates with changes in fatty acid uptake or utilization. The precise nature of its involvement has, however, not been fully defined. FABP may also serve to control the compartmentalization of fatty acids and its derived acyl-CoA esters. In this respect, it is interesting that Capron *et al.* (1979) observed by immunocytochemical techniques that the protein has not a uniform distribution in liver cells, but instead shows a preferential localization around the mitochondria and the membranes of the smooth endoplasmic reticulum. Subcellular distribution studies revealed that in rat intestinal mucosa (Ockner & Manning, 1974) about 15% and in rat liver (Conneely *et al.*, 1983) about 40% of the cellular FABP is membrane-associated.

## 1.6. OUTLINE OF THE PRESENT STUDY

The aim of our investigation was to obtain further insight into various aspects of fatty acid oxidation in muscle. First, we compared the

use of cell-free and cellular systems for the study of fatty acid oxidation in rat skeletal muscle as well as in human and rat heart, and examined the postnatal development of the capacity of fatty acid oxidation in several types of rat muscle. The second part of the thesis deals with studies on the assay and purification of fatty acid-binding protein and its role in cellular fatty acid metabolism.

In a previous study from this laboratory (Thesis Van Hinsbergh, 1979) a sensitive and accurate assay was developed for the determination of long-chain fatty acid oxidation rates with isolated muscle mitochondria. We examined the application of this assay for muscle whole homogenates, intact muscular preparations and isolated rat heart cells, and subsequently compared various aspects of fatty acid oxidation in these systems (chapters 3 and 7). In particular, with the more complex cellular systems, we determined the influence of fatty acids of endogenous origin on the oxidation of exogenous palmitate. Cell-free and cellular systems were applied for the study of the postnatal development of fatty acid oxidation in rat skeletal and cardiac muscle (chapter 4). We elucidated the nature and origin of β-oxidation intermediates that were previously observed to accumulate during palmitate oxidation in cell-free systems (chapter 5). Because of contradictory reports on the effect of clofibrate administration on muscle fatty acid oxidation, we reevaluated and compared the effect of this drug on the palmitate oxidation capacity of rat skeletal muscle and liver (chapter 6).

For the study of the fatty acid-binding protein it was necessary to develop a more adequate procedure for the detection and assay of this protein (chapter 8). The material applied for the latter procedure appeared also suitable for the delipidation of serum albumin (chapter 9). In chapter 10 we estimated the content of the fatty acid-binding protein in rat heart and liver cytosol and investigated its diurnal rhythm. Subsequently, the fatty acid-binding protein was purified and partially characterized from rat heart and liver (chapter 11).

In chapter 12 a general survey and summary of the results will be given.

# CHAPTER 2

# GENERAL EXPERIMENTAL PROCEDURES

## 2.1. SOURCE OF TISSUES

*Animals*

Random-bred albino rats of the Wistar strain were used. The animals were males, weighing either 90-120 or 180-240 g, except when their peri- and postnatal development was studied (chapter 4). Animals were fed with a pelleted diet, containing 22% protein, 4.8% fat and 67% carbohydrates. For clofibrate treatment (chapter 6), rats were orally given 50 mg clofibrate/day in 1 ml 60% (v/v) glycerol solution for 7-10 days. For cholestyramine treatment (chapter 10), rats were fed with a pelleted diet, containing 1% (w/w) cholestyramine, for 5-7 days, as described by Kempen *et al.* (1982). Rats were maintained on a cycle of alternating 12-h periods of light and darkness (light on 7.00 a.m., light off 7.00 p.m.) and were sacrificed by cervical dislocation, routinely between 8.30 and 10.00 a.m., unless the effect of the diurnal rhythm was being investigated (chapter 10).

*Human muscle biopsies*

Biopsies of human m.pectoralis were obtained from breast carcinoma patients. Within 15 min after breast amputation the biopsies were transferred to ice-cold SET-buffer (0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH 7.4). Subsequent transport of the material to the laboratory usually took 1-2 h.

Biopsies (200-500 mg) of the right auricle of adult human heart were obtained during open-heart surgery before cannulation. Immediately after dissection the biopsies were transported to the laboratory (within 5 min, on ice) and washed in calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2), gassed with $O_2/CO_2$ (95:5). Residual fat and connective tissue were removed.

## 2.2. BIOLOGICAL PREPARATIONS

*Intact muscular preparations*

Several types of striated muscular tissue from both rat and man were used. Rat diaphragms were rapidly excised and washed in calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2), gassed with $O_2/CO_2$ (95:5). After removal of residual fat and connective and nerve tissue, the diaphragm was hemisected. Rat m.soleus and m.flexor digitorum brevis were exposed and dissected free by cutting the tendons at their points of insertion. The biopsies of human m.pectoralis were transferred to calcium-free Krebs-Ringer bicarbonate buffer and portions of about 100 mg of teased fibers were prepared.

Rat hearts were rapidly excised, cut into halves and blotted. Heart slices were prepared by dividing the human heart biopsies or the halves of isolated rat hearts into oblong pieces of about 50 mg. The isolation of rat cardiac myocytes is described in chapter 7.

*Preparation of whole homogenates and mitochondria*

After excision the tissues were immediately cooled in SET-buffer. All subsequent procedures were performed at $0^{o}C$. The tissues were minced with a pair of scissors and whole homogenates (10%, w/v, for human m.pectoralis and rat m.quadriceps and m.flexor; 5%, w/v, for all other tissues) were prepared in the same buffer by hand homogenization using a teflon-glass Potter-Elvejhem tissue homogenizer and two pestles with different diameter (the intervening space was 0.12 and 0.05 mm, respectively). In case of m. flexor homogenization was performed with a Polytron tissue homogenizer.

For preparation of mitochondria, the homogenates were centrifuged for 5 min at 600 x *g* and the resulting supernatants (termed postnuclear fraction) for 10 min at 7000 x *g*. The mitochondria were washed with a solution of 100 mM KCl, 50 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 0.2 mM ATP and 0.5% (w/v) fatty acid-free albumin, and finally suspended in the same solution without albumin. The yield of mitochondria, isolated by this procedure, was 10-20% for heart and skeletal muscle. The preparations will predominantly contain mitochondria from subsarcolemmal origin, since no proteolytic enzyme was used.

*Preparation of rat liver and heart cytosol*

Rats were killed by cervical dislocation or anesthesized with diethylether. After severing the vena cava inferior, the liver was perfused *in situ* through a catheter in the vena portae for 2 min with ice-cold buffer, consisting of 100 mM sucrose, 50 mM KCl, 30 mM EDTA and 50 mM K-phosphate (pH 7.2), or with 10 mM K-phosphate buffer (pH 7.4) containing 154 mM NaCl. During perfusion the liver was gently massaged. Rat hearts were similarly perfused through a catheter inserted directly into the right ventricle with the perfusate emerging through the cut cerebral arteries.

After excision the tissues were immediately cooled in ice-cold perfusion buffer. A 25% (w/v) homogenate was prepared in the same buffer by hand homogenization at 0°C using a teflon-glass Potter-Elvejhem tissue homogenizer. The homogenate was centrifuged for 10 min at 600 x *g* and 4°C, and the resulting supernatant was centrifuged for 90 min at 105,000 x *g* and 4°C in a Beckman L2-65 B ultracentrifuge, using rotor 50 Ti. The clear 105,000 x *g* supernatant fraction (cytosol) was aspirated in order to minimize contamination by floating fat and stored at -20°C.

## 2.3. BIOCHEMICAL METHODS

*Cytochrome c oxidase assay*

Cytochrome *c* oxidase (EC 1.9.3.1) activity was assayed by the method of Smith (1955) as modified by Van Hinsbergh *et al.* (1978a). Before the assay mitochondrial membranes were broken by 4 cycles of freezing and thawing. Preparations of freshly isolated mitochondria and rat cardiac myocytes were diluted with 9 volumes of SET-buffer and then frozen and stored at -20°C. Assays were always carried out within two weeks after preparation. Activity of cytochrome *c* oxidase was determined spectrophotometrically at 25°C in 50 mM K-phosphate buffer (pH 7.4) by measuring the decrease of reduced cytochrome *c* at 550 nm. The concentration of cytochrome *c* was 90 µM. All results are expressed in units of enzyme (µmol cytochrome *c* oxidized per min at 25°C).

*Citrate synthase assay*

Citrate synthase (EC 4.1.3.7) activity was measured in supernatants of sonicated preparations according to the method of Shepherd & Garland (1969). The reaction involved is:

$$\text{Acetyl-CoA} + \text{oxaloacetate} \xrightarrow{\text{citrate synthase}} \text{citrate} + \text{CoA-SH}$$

All preparations were stored as described for the assay of cytochrome *c* oxidase. After sonication (Branson sonifier, set at 45 W, 4 x 15 s at 0°C), the preparations were centrifuged for 5 min at 10,000 x *g* and 4°C in a Burkard Koolspin microcentrifuge. Aliquots of the supernatant were used for the assay. The method is based on the chemical coupling of the released CoA-SH to 5,5'-dithiobis-(2-nitrobenzoic acid), which can be traced by the formation of the absorbing mercaptide ion at 412 nm. All results are given in units of enzyme (µmol coenzyme A liberated per min at 25°C).

*Creatine kinase assay*

Creatine kinase (EC 2.7.3.2) activity was measured at 37°C with the CK *N*-acetyl-L-cystein-activated monotest (Boehringer, Mannheim, F.R.G.), in which by a coupled assay procedure the formation of NADPH is traced at 340 nm. Whole homogenates (1%, w/v) of fresh or incubated muscular preparations were prepared in SET-buffer, as described above. The homogenates were centrifuged for 5 min at 10,000 x *g* and aliquots of the supernatant were used for the assay. Creatine kinase activity was also measured in 10-µl samples withdrawn from the incubation media after certain time intervals. All results are given as relative activities or in units of enzyme (µmol/min at 37°C).

*Glycerol determination*

Glycerol was determined with the ultraviolet method, using a Boehringer Test Combination (Boehringer, Mannheim, F.R.G.). With this method the amount of glycerol is, by a coupled assay procedure, reflected in the amount of NADH consumed. NADH is determined by means of its absorption at 340 nm. Before the glycerol determination, protein was precipitated by addition of 0.5 M perchloric acid at 0°C and centrifugation for 5 min at 10,000 x *g*. Subsequently, the supernatants were neutralized with $KOH/K_2HPO_4$.

*Free fatty acid determination*

The total amount of long-chain free fatty acids (FFA) and phospholipids were extracted from the protein sample with Dole's extraction mixture (isopropanol/heptane/0.5 M sulfuric acid, 40:10:1, by vol) (Dole & Meinertz, 1960). Phospholipids were then separated from the long-chain fatty acids by binding to silicic acid (1 g per 40 ml of the heptane/isopropanol phase).

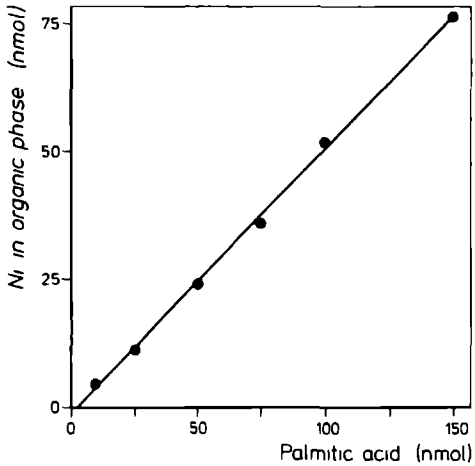



Fig. 2.1. Standard curve of palmitic acid determination with $^{63}$Ni as tracer. The results are shown of a representative experiment out of four.

Thereafter, the long-chain FFA were assayed radiochemically with the use of $^{63}$Ni as tracer, as described by Ho (1970). With standard solutions of palmitic acid in heptane we found a fatty acid to Ni ratio of 1.95 ± 0.07 (mean ± S.D. of four determinations) (Fig. 2.1), which corresponds to the formation of a Ni(fatty acid)$_2$ complex (Ho, 1970; De Brabander & Verbeke, 1981). The recovery of fatty acids from the extraction procedure was determined by adding a known amount of palmitic acid in heptane solution to the extraction mixture containing the protein sample, and was found to be 85-95%.

*Protein determination*

Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

## 2.4. MATERIALS

The specific reagents and materials used in this study are listed below together with their suppliers.
[1-$^{14}$C]- and [U-$^{14}$C]palmitic acid, [1-$^{14}$C]oleic acid, [1-$^{14}$C]succinate, L-[1-$^{14}$C]malate, $^{63}$NiCl$_2$, *N*-[$^{14}$C]methylated bovine serum albumin and ovalbumin: Amersham International, U.K.
[1-$^{14}$C]Myristic acid, [1-$^{14}$C]lauric acid and [3-$^{14}$C]palmitic acid: Applied

Science Europe, Oud-Beyerland, The Netherlands.

[16-$^{14}$C]Palmitic acid and Omnifluor: New England Nuclear, Dreieichenhain, F.R.G.

Human, bovine and rat serum albumin (fraction V), horse heart and skeletal muscle myoglobin, phosphorylase a, leucine aminopeptidase, ovalbumin, α-chymotrypsinogen and malonyl-CoA: Sigma Chemical Company, St. Louis, MO, U.S.A.

Horse heart cytochrome *c*, coenzyme A, acetyl-CoA, ADP, ATP, $NAD^+$ and antimycin A: Boehringer, Mannheim, F.R.G.

L-Carnitine: Sigma Tau Ltd., Rome, Italy.

D,L-Carnitine: Fluka, Buchs, Switzerland.

Silicic acid: Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.

Active charcoal, Coomassie brilliant blue R-250 and silica gel 60 H plates: Merck, Darmstadt, F.R.G.

Clofibrate (Atromid S): ICI, Rotterdam, The Netherlands.

Cholestyramine (Questran): Bristol Myers, Weesp, The Netherlands.

Collagenase (type II): Worthington Biochemical Corporation, Freehold, NJ, U.S.A.

Aqualuma: J.T. Baker, Deventer, The Netherlands.

Lipidex 1000 and Opti-Fluor: Packard Instrument Company, Inc., Downers Grove, IL, U.S.A.

Sephadex G-75, DEAE-Sephacel, Protein A-Sepharose CL-4B, Percoll and Dextran T-70: Pharmacia Fine Chemicals, Uppsala, Sweden.

Rabbit anti-rat albumin antiserum: Nordic Immunology, Tilburg, The Netherlands.

Albumin was freed of fatty acids by acid-charcoal treatment according to the method of Chen (1967) or by Lipidex chromatography at 37°C (chapter 9), dialyzed and lyophilized at neutral pH.

Silicic acid was washed successively with petroleum benzine (boiling-range 40-60°C), chloroform, methanol and acetone, and was activated by heating for 16 h at 120°C.

All other chemicals were of the purest grade commercially available.

# CHAPTER 3

# PALMITATE OXIDATION BY INTACT PREPARATIONS AND WHOLE HOMOGENATES OF SKELETAL MUSCLE*

## 3.1. INTRODUCTION

The important role of fatty acids as fuel for the energy production in skeletal muscles is well established, although it is still not known to what extent fatty acids are preferred over other substrates under given conditions. It has been suggested that fatty acid oxidation may contribute from 20 to 80% of the energy production (Fritz *et al.*, 1958; Bylund *et al.*, 1975; Hagenfeldt, 1979). The supply of fatty acids to muscle can vary largely depending on the nutritional state (Wang *et al.*, 1979; Brass & Hoppel, 1981), exercise (Carlson *et al.*, 1971; Hagenfeldt, 1979) and pathological conditions. Fatty acids can be supplied by serum albumin to which they are non-covalently bound, or else by the action of lipoprotein lipase on serum lipoproteins (Mackie *et al.*, 1980). Uptake of free fatty acids by muscle is linearly related to their arterial concentration (Hagenfeldt, 1979). Fatty acid binding to albumin is less tight at higher fatty acid:albumin molar ratios (Spector & Fletcher, 1978). Reduction of the fatty acid:albumin ratio is accompanied by a proportional reduction in myocardial uptake of fatty acids (Miller *et al.*, 1976).

Many studies dealing with fatty acid oxidation have employed cell-free systems of muscle, while less information is available on oxidation in muscle preparations that contain intact cells (Fritz *et al.*, 1958; Neptune *et al.*, 1959; Fritz & Kaplan, 1960; Schwartzman & Brown, 1960; Eaton & Steinberg, 1961; Bodel *et al.*, 1962; Schonfeld & Kipnis, 1968; Bylund *et al.*, 1975; Tutwiler, 1978; Stearns *et al.*, 1979; Pearce & Connett, 1980; Okano & Shimojo, 1982). In addition, with these preparations fatty acid oxidation was always calculated from the liberation of $^{14}CO_2$ from $^{14}C$-labeled fatty

*Adapted and extended from Glatz & Veerkamp (1982b).

acids alone, although measurement of the sum of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products is a much more sensitive and accurate method (Van Hinsbergh *et al.*, 1978a,b). Therefore, we have first reevaluated the conditions for the *in vitro* determination of the palmitate oxidation rates by preparations of intact muscle. In addition, we have paid special attention to the effects of the palmitate concentration and the palmitate:albumin molar ratio. For comparison, palmitate oxidation rates were also determined in whole homogenates of muscle. The results indicate that the oxidation rate of exogenous palmitate in intact muscle greatly depends on the medium concentration of the unbound substrate and on the amount of free fatty acids derived from lipolysis of endogenous triacylglycerols.

## 3.2. MATERIALS AND METHODS

Male random-bred albino Wistar rats weighing 90-120 g were used. Unless the effect of starvation were being studied the animals were starved for 18 h. Intact muscular preparations were obtained and handled and whole homogenates were prepared as described in section 2.2.

*Assay of palmitate oxidation*

The intact muscular preparations were gently blotted on filter paper, weighed and immediately transferred to 20-ml incubation vials containing calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2) gassed with $O_2/CO_2$ (95:5), supplemented with 4 mM D,L-carnitine and 0.1 mM coenzyme A. The final incubation volume was 2.5 ml (hemidiaphragm and m.pectoralis fibers), 1 ml (m.soleus) or 0.5 ml (m.flexor), giving 40-50 mg muscle/ml in all cases. The vials were sealed with rubber caps and equipped with a disposable center well. After 5 min preincubation at 37°C the reaction was started by the addition of [$^{14}C$]palmitate bound to dialyzed, fatty acid-free albumin in various molar ratios. The incubation proceeded at 37°C for 60 min (m.pectoralis) or 90 min (all other tissues) and was terminated by the addition of 0.5 ml 3 M perchloric acid to the medium, followed by a second incubation for 18 h at 4°C. $^{14}CO_2$ produced was trapped in 0.3 ml ethanolamine/ethyleneglycol (1:2, v/v) injected into the center well. Thereafter the acid incubation mixture was centrifuged in a Burkard Koolspin microcentrifuge and an aliquot of the supernatant was assayed for radioactivity by liquid scintillation counting in Aqualuma. Radioactivity of $^{14}CO_2$ was

measured in 10 ml toluene/methanol (2:1, v/v) containing 0.4% Omnifluor. Palmitate oxidation rates were calculated from the production of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products and are expressed as nmol/min per g muscle (wet weight). To exclude biological variations the effect of certain metabolites was always tested with one hemidiaphragm or leg muscle, while contralateral muscles from the same rat were used as paired controls. In all experiments the two similar muscular preparations from one rat were never used for testing the same experimental condition.

With homogenates the palmitate oxidation rate was measured in a medium containing 0.25 M sucrose, 30 mM KCl, 10 mM K-phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 75 mM Tris-HCl (pH 7.4) supplemented with 5 mM ATP, 1 mM $NAD^+$, 25 µM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. The final incubation volume was 0.5 ml containing 50-100 µl homogenate (2.5 - 5 mg muscle). Incubations were carried out with 120 µM [1-$^{14}C$]-palmitate bound to albumin (molar ratio 5:1) essentially as described above except that the incubation time was 30 min.

*Lipolysis studies*

Hemidiaphragms were incubated for 30 min under the same conditions as described for the assay of palmitate oxidation. The glycerol and free fatty acids released into the medium were measured in separate aliquots taken at the end of each incubation and kept frozen at -20°C until analyzed. Glycerol was determined as described in section 2.3. Free fatty acids were extracted according to the method of Dole & Meinertz (1960) and assayed radiochemically with the use of $^{63}Ni$ as tracer (Ho, 1970) as described in section 2.3. For identification, the fatty acids were, after extraction, methylated with borium trifluoride in methanol (Morrison & Smith, 1964) and determined by gas-liquid chromatography (Veerkamp, 1970).

*Other assays*

Creatine kinase and citrate synthase activities were measured as described in section 2.3.

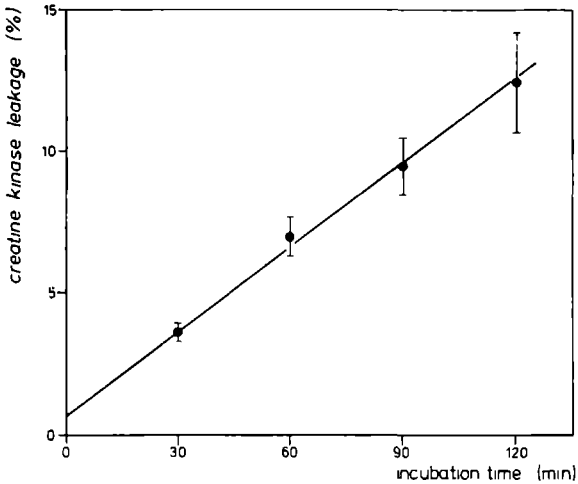



Fig. 3.1. Leakage of soluble creatine kinase into the medium during incubation of rat hemidiaphragm in the presence of 4 mM D-glucose. Values represent means ± S.D. of 6 preparations.

## 3.3. RESULTS

*Intactness of the muscular preparations*

To estimate the intactness of the muscular preparations we measured the leakage of soluble (cytosolic) creatine kinase during incubation in the presence of 4 mM D-glucose as the only substrate. Upon incubation of hemidiaphragm the activity appearing in the medium increased linearly with time (Fig. 3.1). After 90 min creatine kinase leakage amounted to 9.5 ± 1.0% (mean ± S.D. of 6 preparations). The sum of the activities present in the incubation medium and recovered in the hemidiaphragm after incubation was always equal to the activity of the non-incubated preparation of the counter hemidiaphragm. This indicates that the creatine kinase activity of the incubation medium was not underestimated due to proteolytic activity or some inhibitor. The total cellular creatine kinase activity, measured in 10,000 x *g* supernatants of sonicated whole homogenates of diaphragm was 4260 ± 400 mol/min per g, of which 91 ± 1% resides in the cytosol (means ± S.D. of 6 preparations). The latter value corresponds to data of Scholte (1973a) on rat masseter muscle. Upon incubation of m.soleus and m.flexor for 90 min and m.pectoralis for 60 min leakage of creatine kinase was 2.1 ± 0.4, 1.7 ± 0.8 and 13.9 ± 1.0%, respectively (means ± S.D. of 4 preparations). From these observations it is concluded that all muscular prepara-

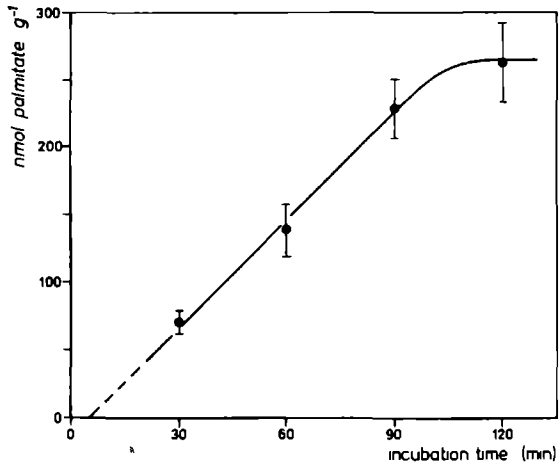



Fig. 3.2. Time dependence of the oxidation of 120 μM [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1) by rat hemidiaphragm. Values represent means ± S.D. of 10-12 preparations.

tions can be regarded as remaining reasonably intact during incubation.

*Palmitate oxidation by intact preparations and the effect of various factors*

With hemidiaphragm palmitate oxidation appeared to be linear with time for at least 90 min with a lag period of about 5 min (Fig. 3.2.). This lag period probably reflects the time required for steady-state isotopic labeling of the intracellular space and metabolic pathways. A similar time dependency was observed with m.soleus and m.flexor (data not shown). With teased fibers of human m.pectoralis linearity was observed for 60 min. Some authors preincubate hemidiaphragms at 37°C in buffer containing 4 mM D-glucose to allow the preparation to recover from the hypoxic stress of the dissection (Goldberg *et al.*, 1975; Pearce & Connett, 1980). After 30 min preincubation the preparations showed the same oxidation rates and linearity with time when compared to those that were not preincubated. Therefore, preincubation was omitted in all further experiments.

Palmitate oxidation was linear with the amount of tissue material (up to 150 mg). No differences were observed between the oxidation rates of left and right hemidiaphragms, when incubated under the same conditions (data not shown). Like with cell-free preparations (Van Hinsbergh *et al.*, 1978a,b), palmitate oxidation rates can also be more accurately and sensitively be measured in intact muscular preparations from the sum of $^{14}CO_2$ and $^{14}$C-la-

beled acid-soluble products than from $^{14}CO_2$ production alone. During incubation with [1-$^{14}$C]palmitate $^{14}CO_2$ represented 50-60% of the total acid-soluble products formed (cf. Table 5.2 and Fig. 5.1).

Various metabolites were tested for their effect on palmitate oxidation by hemidiaphragm (Table 3.1). Omission of L-carnitine, with or without coenzyme A, decreased the oxidation rate slightly (by 19%). For economical reasons we used in most experiments 4 mM D,L-carnitine, which has the same effect as 2 mM L-carnitine. Coenzyme A itself had no effect. The oxidation rate was not influenced by the addition of L-malate, pyruvate or D-glucose, but was slightly decreased by $Ca^{2+}$. The absence of an effect of D-glucose on palmitate oxidation is consistent with results of other studies (Fritz & Kaplan, 1960; Schwartzman & Brown, 1960; Eaton & Steinberg, 1961). Glucose

*Table 3.1. Effect of various factors on palmitate oxidation by rat hemidiaphragm.*

| Addition ( + ) or omission ( - ) | Relative activity |
|---|---|
| None | 100 |
| - 0.1 mM CoASH | 100 ± 9 (7) |
| - 4 mM D,L-carnitine | 81 ± 4 (4) |
| - 0.1 mM CoASH - 4 mM D,L-carnitine | 82 ± 7 (6) |
| - 4 mM D,L-carnitine + 2 mM L-carnitine | 101 ± 8 (4) |
| + 5 mM ATP | 124 ± 22 (7) |
| + 5 mM adenosine | 79 ± 2 (3) |
| + 1 mM L-malate | 104 ± 3 (3) |
| + 1 mM pyruvate | 100 ± 1 (3) |
| + 4 mM D-glucose | 92 ± 3 (4) |
| + 0.25 mM $Ca^{2+}$ | 94 ± 10 (5) |
| + 2.5 mM $Ca^{2+}$ | 80 ± 16 (5) |
| - 0.1 mM CoASH - 4 mM D,L-carnitine + 5 mM ATP | 85 ± 4 (3) |
| - 0.1 mM CoASH - 4 mM D,L-carnitine + 1 mM L-malate | 85 ± 3 (3) |

Standard medium contained 4 mM D,L-carnitine, 0.1 mM CoASH and 120 µM [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1). Values (in % of the control) are means ± S.D. of the number of paired experiments given within parentheses.

markedly stimulates, however, the rate of incorporation of palmitate into muscle triacylglycerol (Eaton & Steinberg, 1961). ATP has a small and variable stimulatory effect on palmitate oxidation, but only in the presence of carnitine and coenzyme A. This effect may relate to palmitate activation in some cells that are damaged during preparation of the muscles. The stimulation is not due to adenosine supply, since this substance has an opposite effect.

The stimulation of the oxidation rate by carnitine relates to the degree of injury of the muscular preparation, since this stimulation was markedly enlarged when the hemidiaphragm preparation was plurally incised (Fig. 3.3). It is noteworthy that the oxidation rate itself is not dependent on the degree of injury, if carnitine is present. Furthermore, a stimulation of the same order of magnitude was also observed with plurally incised m.soleus, while carnitine did not affect the palmitate oxidation by intact m.soleus (Fig. 3.3). Also, the oxidation rate of carnitine-stimulated incised m.soleus is comparable to that of intact m.soleus. Thus, carnitine appears to be the main coenzyme of palmitate oxidation that is lost upon injury of the muscular preparation. Efflux of carnitine was observed with rat extensor digitorum longus (Rebouche, 1977). When measured in the absence of carnitine, the ratio between the production of $^{14}CO_2$ and $^{14}C$-labeled

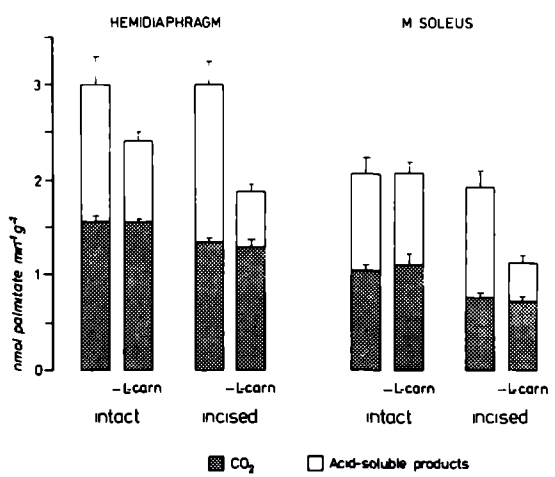



Fig. 3.3. Effect of 2 mM L-carnitine on the oxidation of 120 μM [1-$^{14}C$]-palmitate bound to albumin (molar ratio 5:1) by intact or incised rat hemidiaphragm and m.soleus. Values are means ± S.D. of 4 preparations.

*Table 3.2. Palmitate oxidation rates of intact muscles and whole homogenates of young rats and the effect of starvation.*

| Muscle and condition | Muscle weight | Palmitate oxidation rate nmol/min | | | Citrate synthase activity |
|---|---|---|---|---|---|
| | | Intact muscle | Whole homogenate | | |
| | mg | /g muscle | /g muscle | /unit citrate synthase | unit/g muscle |
| Human m.pectoralis | - | 4.38 ± 1.51 | 29.8 ± 6.7 | - | 5.3 ± 1.4 |
| Rat diaphragm | | | | | |
| Fed | 214 ± 17 | 2.47 ± 0.14 | 390 ± 42 | 11.59 ± 1.46 | 33.9 ± 3.2 |
| Starved 18 h | 215 ± 6 | 2.83 ± 0.38 | 371 ± 42 | 11.28 ± 1.13 | 32.9 ± 1.0 |
| 42 h | 185 ± 14 | 2.92 ± 0.51 | 331 ± 35 | 11.02 ± 0.92 | 30.0 ± 2.6 |
| 66 h | 172 ± 15 [a] | 4.01 ± 0.75 [a] | 305 ± 68 [b] | 10.57 ± 2.29 | 29.1 ± 4.2 |
| Rat m.soleus | | | | | |
| Fed | 74.7 ± 3.5 | 2.38 ± 0.68 | 288 ± 30 | 9.04 ± 0.28 | 32.8 ± 4.0 |
| Starved 18 h | 71.1 ± 2.9 | 2.13 ± 0.26 | 301 ± 43 | 8.93 ± 0.89 | 33.6 ± 2.4 |
| 42 h | 68.2 ± 5.8 | 1.93 ± 0.28 | 248 ± 14 | 9.12 ± 1.05 | 26.7 ± 3.0 |
| 66 h | 67.5 ± 2.8 [b] | 3.21 ± 0.72 [b] | 223 ± 31 [b] | 9.34 ± 0.57 | 23.9 ± 3.5 [a] |
| Rat m.flexor digitorum brevis | | | | | |
| Fed | 43.6 ± 4.2 | 2.16 ± 0.20 | 25.9 ± 6.0 | 5.12 ± 0.96 | 3.7 ± 0.5 |
| Starved 18 h | 47.2 ± 4.0 | 1.72 ± 0.20 | - | - | - |
| 42 h | 46.8 ± 8.4 | 1.78 ± 0.11 | - | - | - |
| 66 h | 40.0 ± 5.1 | 1.95 ± 0.24 | - | - | - |

Oxidation rates were determined with 120 μM [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1). In case of intact muscles one hemidiaphragm or leg muscle was incubated per vial. The oxidation rate of each m.pectoralis was the mean of three separate fiber preparations. Muscle weights given are those of the entire diaphragm or of both leg muscles. In separate experiments palmitate oxidation rates and citrate synthase activities were measured in whole homogenates which were prepared from entire diaphragm or from both leg muscles. At the beginning of starvation all rats used weighed 110-120 g. After 18, 42 and 66 h of starvation, rat weights amounted to 101 ± 4, 91 ± 6 and 83 ± 4 g, respectively (means ± S.D. of 4 animals). All values represent means ± S.D. of 4-14 experiments. Values obtained with preparations from fed rats are compared with those from rats starved for 66 h by the unpaired *t*-test.

[a] $P < 0.01$

[b] $P < 0.05$

acid-soluble products appears to correlate with the degree of injury of the muscular preparation, since with intact hemidiaphragm and m.soleus this ratio amounted to 1.50 ± 0.15 and 1.11 ± 0.14 and with incised hemidiaphragm and m.soleus to 2.01 ± 0.18 and 1.62 ± 0.20, respectively (means ± S.D., 3-6 experiments). Therefore, intactness may also be judged from the relative contributions of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products to the total palmitate oxidation rate in these muscular preparations.

*Comparison of oxidation rates of intact preparations and whole homogenates and the effect of starvation*

With intact muscular preparations from fed animals the palmitate oxidation rates did not differ among the muscles examined (Table 3.2). After starvation of the animals for 66 h the oxidation rates were 40-60% higher with hemidiaphragm and m.soleus but remained unaltered with m.flexor. With whole homogenates we measured palmitate oxidation at cofactor and substrate concentrations that gave maximal oxidation rates. The oxidation rates of homogenates of diaphragm and m.soleus, expressed per g muscle, are comparable and more than 10-fold higher than those of m.flexor or human m.pectoralis. Upon starvation for 66 h they decrease by about 20% (Table 3.2). However, when the oxidation rates are expressed relative to the citrate synthase activity, there is no significant decrease with starvation (Table 3.2).

Because m.flexor contains comparatively large amounts of connective tissue it is difficult to homogenize this muscle by the Potter-Elvejhem method. Therefore, we used a Polytron tissue homogenizer. To check if this treatment injures muscle mitochondria, we measured palmitate oxidation rates and the activities of cytochrome *c* oxidase and citrate synthase of homogenates of diaphragm, prepared by either the Potter-Elvejhem or the Polytron method. The ratios of activities (Polytron relative to the Potter-Elvejhem method) were 0.98 ± 0.03, 0.63 ± 0.08 and 1.10 ± 0.18 for palmitate oxidation, cytochrome *c* oxidase and citrate synthase, respectively (means ± S.D., 4 hemidiaphragms, tested in paired experiments). It appears that in the mitochondria from the Polytron treatment the membrane-associated enzyme cytochrome *c* oxidase loses part of its activity. However, this activity still seems high enough to enable palmitate oxidation to proceed unaltered. The palmitate oxidation rates measured in Polytron homogenates of m.flexor can, therefore, be compared with those of the other muscles.

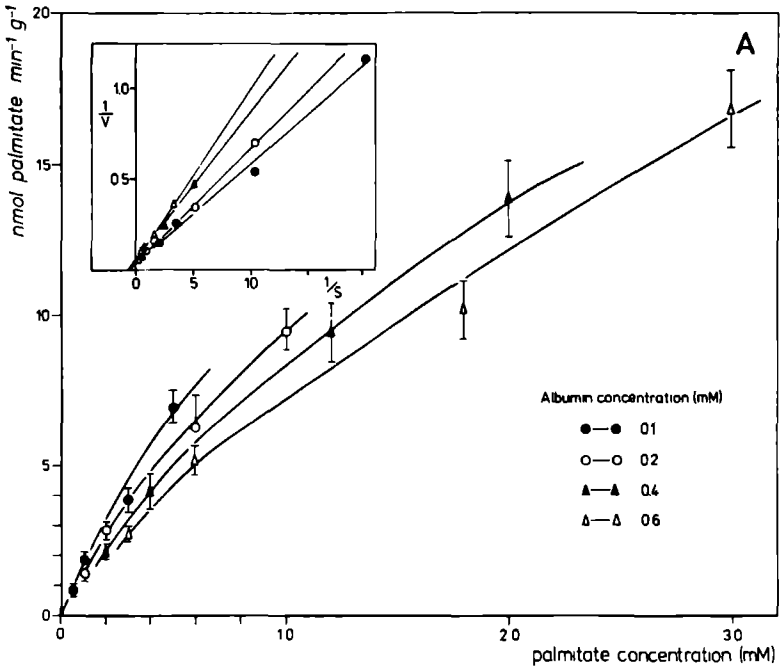



Fig. 3.4. Concentration dependence of palmitate oxidation by rat hemidiaphragm for several albumin concentrations (A) and for several palmitate:albumin molar ratios (B; facing page). Values are means ± S.D. of 6-14 hemidiaphragms. In (A) the inset figure shows the reciprocal of the palmitate oxidation rate versus the reciprocal of the palmitate concentration. The inset figure in (B) shows the S.D. for 0 - 0.5 mM palmitate. All concentrations were corrected for substrate utilization according to the method of Lee & Wilson (1971).

*Concentration dependence of palmitate oxidation by hemidiaphragm*

With hemidiaphragm we studied the dependence of the palmitate oxidation rate on external substrate concentration (0-3 mM) at several albumin concentrations (0.1 - 0.6 mM) and palmitate:albumin molar ratios (0.5 - 5.0). Throughout this range of physiologically relevant concentrations the oxidation rate appeared to increase with both the palmitate concentration and the palmitate:albumin molar ratio (Fig. 3.4). For all albumin concentrations examined we calculated the maximal oxidation rates and apparent $K_m$ values (Table 3.3) from the corresponding Lineweaver-Burk plots (inset of Fig 3.4 A). Linear-regression analysis revealed high correlation coefficients and maximal oxidation rates that did not show much dependence on the

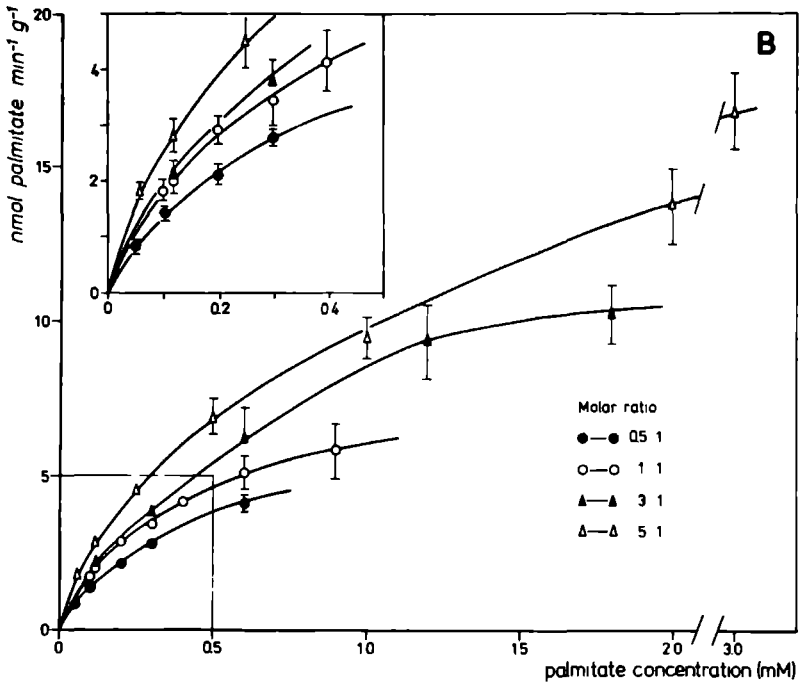



*Table 3.3. Kinetic parameters of the concentration dependence of palmitate oxidation by rat hemidiaphragm.*

| Condition | Albumin concentration (mM) | Apparent $K_m$ (mM) | $V_{max}$ (nmol/min per g) | Correlation coefficient |
|---|---|---|---|---|
| Starved 18 h | 0.10 | 1.64 | 29.6 | 0.98 |
| | 0.20 | 1.43 | 22.6 | 0.99 |
| | 0.40 | 2.76 | 31.9 | 0.98 |
| | 0.60 | 2.70 | 27.9 | 0.97 |
| Starved 66 h | 0.40 | 0.98 | 24.8 | 0.95 |

Parameters of each linear regression line were calculated with the aid of the individual oxidation rates of all hemidiaphragms corresponding to the specific concentration curve (visualized in the inset of Fig. 3.4 A and in Fig. 3.5). For each curve 30-36 hemidiaphragms, distributed over 4 different palmitate concentrations, were used.

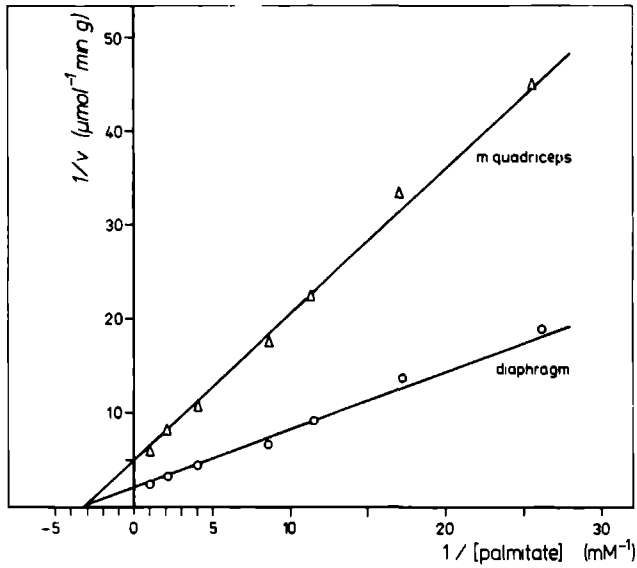



Fig. 3.5. Lineweaver-Burk plots of the concentration dependence of palmitate oxidation by whole homogenates of diaphragm (o) and m.quadriceps (Δ), measured at 0.4 mM albumin. The results are shown of a typical experiment with homogenates of muscles obtained from the same rat. All concentrations were corrected for substrate utilization according to the method of Lee & Wilson (1971).

albumin concentration (23-32 nmol/min per g). At 0.1 and 0.2 mM albumin the apparent $K_m$ was, however, lower than at 0.4 and 0.6 mM albumin (Table 3.3).

For comparison we also measured the concentration dependence of palmitate oxidation in whole homogenates of diaphragm. At 0.4 mM albumin the apparent $K_m$ was 372 ± 98 μM and the maximal oxidation rate 701 ± 140 nmol/min per g (means ± S.D., 4 experiments with rats that had been starved for 18 h) (Fig. 3.5). With whole homogenates of rat m.quadriceps we observed under these conditions a similar apparent $K_m$, but a much lower maximal oxidation rate (360 ± 88 μM and 249 ± 30 nmol/min per g, respectively; means ± S.D., 4 experiments).

In order to determine the extent to which endogenous fatty acids may influence the oxidation of exogenous palmitate in diaphragm, we have measured the effect of the nutritional state on the kinetic parameters of palmitate oxidation and also the rate of endogenous fatty acid consumption. After starvation the apparent $K_m$ for palmitate oxidation by hemidiaphragm decreased considerably, while the maximal oxidation rate hardly changed (Fig. 3.6). Glycerol release increased linearly with time and was not mar-

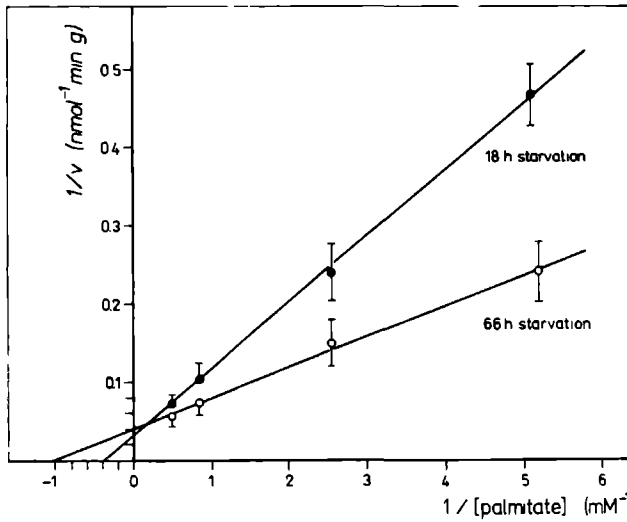



Fig. 3.6. Effect of prolonged starvation on the concentration dependence of palmitate oxidation by rat hemidiaphragm at 0.4 mM albumin. Values are means ± S.D. of 6-9 preparations. Rats weighing 110-120 g at the beginning of starvation were starved for 18 h (•) or for 66 h (o). All concentrations were corrected for substrate utilization according to the method of Lee & Wilson (1971).

kedly changed by the presence of 120 µM palmitate bound to albumin (molar ratio 5:1), or of 0.4 mM albumin. Hemidiaphragm and m.soleus from fed rats showed comparable rates of glycerol release (Table 3.4), which are slightly less than those observed with rat heart slices (section 7.3). The release of fatty acids to the medium appeared rather variable (Table 3.4) and was 2-3-fold higher in the presence of the palmitate-albumin complex (results not shown). The latter observation may relate to withdrawal of fatty acids from the muscle by albumin. The released fatty acids were palmitic acid (49%), stearic acid (23%) and oleic acid (28%). The palmitate oxidation was also measured in the muscles which were first used for the release experiments. After reincubation a similar oxidation rate was observed as in the non-preincubated muscles. The rate of endogenous fatty acid consumption, calculated from the release of glycerol into the medium and corrected for the release of free fatty acids, amounts to 25-30 nmol/min per g for both hemidiaphragm and m.soleus from fed rats (Table 3.4). This value is of the same order of magnitude as the calculated maximal oxidation rate for hemidiaphragm (Table 3.3).

*Table 3.4. Endogenous free fatty acid consumption by rat hemidiaphragm and m.soleus.*

| Muscle and condition | Glycerol release | Free fatty acid release | Endogenous free fatty acid consumption |
|---|---|---|---|
| Hemidiaphragm | | | |
| Fed | 15.8 ± 1.8 (12) | 16.1 ± 5.6 (6) | 29.9 ± 9.5 (6) |
| Starved 66 h | 12.7 ± 2.8[b] (10) | 15.3 ± 5.8 (4) | 17.6 ± 8.1[a] (4) |
| M.soleus | | | |
| Fed | 11.3 ± 2.0 (8) | 12.4 ± 4.9 (4) | 24.6 ± 7.6 (4) |
| Starved 66 h | 6.2 ± 2.1[c] (4) | 6.8 ± 3.4[a] (3) | 14.0 ± 6.6[b] (3) |

The muscles were incubated for 30 min without added substrate. The glycerol and free fatty acids released into the medium were measured in separate aliquots taken at the end of the incubations. Endogenous free fatty acid consumption was calculated from the release of glycerol and corrected for the release of free fatty acids. All values are given in nmol/min per g muscle (wet weight) and represent means ± S.D. of the number of preparations indicated within parentheses. Values obtained with preparations from fed rats are compared with those from rats starved for 66 h by the unpaired *t*-test.

[a] $P < 0.05$

[b] $P < 0.01$

[c] $P < 0.001$

After prolonged starvation (66 h) the glycerol release was significantly lower in both hemidiaphragm and m.soleus, when compared to fed animals (Table 3.4). The release of free fatty acids also tended to be lower in both muscles, so that the calculated rate of endogenous free fatty acid consumption was after starvation only about 60% of that of muscles from fed rats (Table 3.4).

## 3.4. DISCUSSION

The present findings demonstrate that intact human and rat skeletal muscle *in vitro* oxidizes palmitate at constant and high rates for at least 60 and 90 min, respectively. The oxidation rate is highly dependent upon the fatty acid availability within the substrate concentration range studied. The latter data confirm earlier observations that the production of $^{14}CO_2$ from [1-$^{14}$C]palmitate by rat diaphragm (Fritz *et al.*, 1958; Bodel *et*

*al.*, 1962) and m.extensor digitorum longus (Eaton & Steinberg, 1961) rises with increasing concentration of fatty acids in the medium. The observation that with hemidiaphragm the oxidation rate is hardly influenced by a variety of substances added to the incubation medium (Table 3.1) shows that this muscle preparation retains its viability during incubation. Lundholm *et al.* (1975) also observed that teased human muscle fibers maintain a normal ultrastructure during incubation and that several regulatory systems remain intact. These results indicate that provision of oxygen and substrate by diffusion is sufficient to maintain a steady metabolic rate, which is necessary for the study of fatty acid metabolism.

The palmitate oxidation rates measured by us for intact rat muscle are generally two to four times as high as those reported by others for rat hemidiaphragm (Fritz & Kaplan, 1960; Bodel *et al.*, 1962; Tutwiler, 1978; Stearns *et al.*, 1979; Higgins *et al.*, 1981), m.soleus (Stearns *et al.*, 1979; Pearce & Connett, 1980) and m.extensor digitorum longus (Eaton & Steinberg, 1961). This is due to the fact that these investigators only measured $^{14}CO_2$ production, which merely accounts for about 50% of the oxidation rate (Fig. 3.3). With teased fibers from human skeletal muscle Bylund *et al.* (1975) measured the $^{14}CO_2$ production from [U-$^{14}$C]palmitate and found a lower oxidation rate than our value for human m.pectoralis.

The concentration of free external palmitate is larger at a higher palmitate:albumin molar ratio, since fatty acids are then less tightly bound to albumin (Spector & Fletcher, 1978). This explains our observation that at relatively low albumin concentrations (0.1 and 0.2 mM) the apparent $K_m$ for palmitate oxidation is lower than at high (0.4 and 0.6 mM) albumin concentrations (Table 3.3, Fig. 3.4). However, recent studies indicate that at low fatty acid concentrations the hepatic uptake of long-chain fatty acids is not determined by the concentrations of free or total fatty acid, but by the concentrations of albumin and of the albumin-fatty acid complex (Weisiger *et al.*, 1981). It could be argued that the apparent $K_m$ is associated with palmitate uptake by the muscle rather than with its oxidation. Transport of fatty acids across the muscle plasma membrane may be rate-limiting, since at the same substrate concentration the oxidation rate measured after prolonged starvation is nearly twice as high as for fed animals (Fig. 3.5). There is, however, no evidence that the permeability of the sarcolemmar membrane for fatty acids changes during starvation.

Free fatty acids derived from endogenous lipid stores, mainly intermyofibrillar triacylglycerol pools, can also enter the fatty acid oxidation pathway, thereby causing label dilution of the exogenously added [$^{14}$C]-palmitate and hence underestimation of the fatty acid oxidation rate. The degree of label dilution is dependent upon the concentration of exogenous fatty acid and the rate of endogenous lipolysis. Owing to competitive inhibition, the apparent $K_m$ for the oxidation of exogenous as well as endogenous fatty acids will be lower than the $K_m$ for the oxidation of exogenous palmitate. However, high concentrations of exogenous fatty acids inhibit endogenous lipolysis in rat heart (Crass, 1977) and m.soleus (Pearce & Connett, 1980), probably by product inhibition of the tissue lipase. With 120 µM palmitate we found no inhibition of glycerol release from hemidiaphragm. The inhibition may still complicate measurements of lipolysis and oxidation of external fatty acids. The linearity of the oxidation with time indicates a nearly immediate equilibration of the pools of external and endogenously formed fatty acids.

During prolonged starvation the apparent $K_m$ for palmitate oxidation, when measured in the presence of 0.4 mM albumin, markedly decreased, but the calculated maximal oxidation rate was not significantly changed (Table 3.3). This suggests a more limited availability of endogenous fatty acids after prolonged starvation. The rate of endogenous free fatty acid consumption was indeed lowered after starvation (Table 3.4). These observations may relate to the decrease of the triacylglycerol content of the diaphragm of these young rats upon starvation (Neptune *et al.*, 1959; Masoro, 1967; Abumrad *et al.*, 1978). An increase in $^{14}CO_2$ production from external $^{14}$C-labeled fatty acids was earlier observed in hemidiaphragm after starvation (Schwartzmann & Brown, 1960; Fritz & Kaplan, 1960; Bodel *et al.*, 1962). The low apparent $K_m$ for palmitate oxidation in homogenates of diaphragm can be explained from the negligible label dilution with endogenously derived substrates due to the large medium volume. We conclude that the apparent $K_m$ for the oxidation of exogenous palmitate by intact hemidiaphragm *in vitro* is greatly affected by the degree of label dilution with endogenously derived free fatty acids. These endogenous lipid pools have a physiological importance, since intramuscular triacylglycerol is mobilized during exercise, predominantly in the slow oxidative red fibers (Reitman *et al.*, 1973; Stankiewicz-Choroszucha & Górski, 1978; Jones *et al.*, 1980; Oscai *et al.*, 1982).

Although diaphragm and m.soleus are comprised almost entirely of slow oxidative red fibers (Baldwin *et al.*, 1972; Gould, 1973), whereas m.flexor is a fast-twitch mixed type muscle, there is no great difference in the palmitate oxidation rates of these muscles, when they are obtained from fed animals (Table 3.2). This may be due to a difference in label dilution with endogenous substrates, since in red fibers the triacylglycerol content is much higher (Reitman *et al.*, 1973; Okano *et al.*, 1980) and triacylglycerol-derived fatty acids are relatively more available (Reitman *et al.*, 1973; Stankiewicz-Choroszucha & Górski, 1978; Oscai *et al.*, 1982) than in white fibers. Recently, Okano & Shimojo (1982) have reported higher palmitate oxidation rates for slices of red than of white portions of m.quadriceps, but all of their values are much lower than ours. In diaphragm and m.soleus a decreased mobilization of endogenous fatty acids may result in higher oxidation rates of external substrate after starvation for 66 h, while in m.flexor presumably hardly any change in label dilution occurs.

The 20% decrease observed in the palmitate oxidation rate of whole homogenates of diaphragm and m.soleus from fasted animals (Table 3.2) may be related to a small decrease in mitochondrial content, since no change of palmitate oxidation rates is observed when these rates are expressed relative to the citrate synthase activity. For all muscles examined, the palmitate oxidation rates of intact muscle are much lower than those of whole homogenates. This difference is likely to be due to three factors: (i) an underestimation of the oxidation rate of the intact muscle due to label dilution, (ii) the resting state of these *in vitro* muscular preparations in which they do not use much of their metabolic capacity (Fritz *et al.*, 1958; Taegtmeyer *et al.*, 1980; McLane *et al.*, 1981), and (iii) the use of entire muscles, which have 5-7 times lower [1-$^{14}$C]palmitate oxidation rates than muscle cells isolated from m.flexor by incubation with collagenase and gentle trituration (Zuurveld, J.G.E.M. and Veerkamp, J.H., unpublished data).

In summary, we have described a method for the assay of palmitate oxidation in intact muscle preparations. Several metabolic characteristics of these preparations have been investigated, including the dependence on external substrate concentration, the role of endogenously derived fatty acids, the effect of various cofactors, and the comparison with oxidation measurements in whole homogenates. This study serves as a basis for further investigations of the postnatal development of palmitate oxidation (chap-

ter 4) and of the fatty acid oxidation by cellular preparations of human and rat heart (chapter 7).

## 3.5. SUMMARY

The palmitate oxidation by intact preparations of rat hemidiaphragm, m.soleus and m.flexor digitorum brevis and by teased fibers of human m.pectoralis has been studied.

The structural and metabolic viability of these preparations *in vitro* is shown by a low leakage of soluble creatine kinase, a constant rate of palmitate oxidation and a small stimulatory effect of L-carnitine.

The palmitate oxidation rate of hemidiaphragm increases with the palmitate concentration (0-3 mM) and with the palmitate:albumin molar ratio (0.5 - 5.0). The apparent $K_m$ for palmitate oxidation is about 1.5 mM at 0.1 and 0.2 mM albumin and about 2.7 mM at 0.4 and 0.6 mM albumin, which correlates with the higher affinity of albumin for palmitate at lower palmitate:albumin molar ratios. After prolonged starvation the apparent $K_m$ at 0.4 mM albumin is markedly decreased. In whole homogenates of diaphragm and of m.quadriceps femoralis the apparent $K_m$ is only about 370 μM at 0.4 mM albumin.

The calculated maximal oxidation rate is not significantly different for all albumin concentrations examined (23-32 nmol/min per g), does not change after starvation, and is about equal to the rate of endogenous fatty acid consumption (25-30 nmol/min per g).

Our results suggest that substrate availability is an important factor for the oxidation rate of exogenous palmitate by hemidiaphragm *in vitro* and that the apparent $K_m$ largely depends upon the degree of label dilution by fatty acids of endogenous origin.

# CHAPTER 4

# POSTNATAL DEVELOPMENT OF PALMITATE OXIDATION AND MITOCHONDRIAL ENZYME ACTIVITIES IN RAT CARDIAC AND SKELETAL MUSCLE*

## 4.1. INTRODUCTION

The development of fatty acid oxidation has been studied more extensively in mammalian liver than in muscle. In rat liver the oxidation capacity is higher during the suckling period than prenatally or after weaning and appears to follow changes in dietary fat supply (Bailey & Lockwood, 1973, Foster & Bailey, 1976; Krahling *et al.*, 1979; Chalk *et al.*, 1983). In heart and skeletal muscle the development of anaerobic metabolism has received more attention. Activities of glycogen metabolizing enzymes increase postnatally in rat heart (Novák *et al.*, 1972; Baldwin *et al.*, 1977)and skeletal muscle (Novák *et al.*, 1972; Baldwin *et al.*, 1978; Villa-Moruzzi *et al.*, 1979) and in rabbit skeletal muscle (Smith, 1980) with major changes being completed in 3-4 weeks.

The fatty acid oxidation capacity of heart muscle appears to rise perinatally and during early postnatal development in rat (Wittels & Bressler, 1965) and calf (Warshaw & Terry, 1970), but not in pig (Wolfe *et al.*, 1978). In rat heart the postnatal rise is accompanied by an increase of the activities of several enzymes associated with oxidative metabolism (Lang, 1965; Wittels & Bressler, 1965; Walpurger, 1967; Warshaw, 1972; McMillin-Wood, 1975; Baldwin *et al.*, 1977) and the concentration of cytochromes (Hallman *et al.*, 1972; Kinnula & Hassinen, 1977; Baldwin *et al.*, 1977), carnitine (Wittels & Bressler, 1965; Robles-Valdes *et al.*, 1976; Borum, 1978) and mitochondrial protein (Hallman *et al.*, 1972; Kinnula & Hassinen, 1977; David *et al.*, 1979; Rakusan, 1980). On the basis of morphometric studies it has been reported that the latter is due to an increase of both the number and size of mitochondria (David *et al.*, 1979; Olivetti *et al.*, 1980), and to

*Adapted from Glatz & Veerkamp (1982a).

an increase of only their size (Vitorica *et al.*, 1981). Little is known about changes of fatty acid oxidation in developing skeletal muscle. In porcine leg muscle homogenates the palmitate oxidation rate, based on $^{14}CO_2$ production, increases with the age of the animal (Wolfe *et al.*, 1978). In rat skeletal muscle carnitine content (Borum, 1978) and citrate synthase activity (Baldwin *et al.*, 1978; Kloosterboer *et al.*, 1979) increase during the suckling period. The age dependence of fatty acid oxidation has never been studied in intact muscular preparations.

In order to establish possible developmental differences between various types of muscle, we have measured the palmitate oxidation rate both in intact preparations of diaphragm and m.flexor digitorum brevis and in whole homogenates of heart, diaphragm and m.quadriceps femoralis of rats one day before birth and at different periods (0-65 days) after birth. The activities of the mitochondrial enzymes cytochrome *c* oxidase and citrate synthase have also been determined. The results indicate that during postnatal development the oxidative capacity of all muscles increases considerably, together with development. We have determined the palmitate oxidation rate and cytochrome *c* oxidase and citrate synthase activities in mitochondrial preparations, in order to establish whether this increase is due to an increase in the number of mitochondria and/or to a higher specific activity of the oxidative system.

## 4.2. MATERIALS AND METHODS

### *Animals*

Albino Wistar rats were used in all experiments. At birth the nest size was, if necessary, decreased routinely to eight animals, since growth rate is affected markedly by litter size (Rakusan *et al.*, 1978; Timson, 1982). At regular intervals after birth one animal from each nest was killed. Fetal age was determined by dated matings. Newborn animals (day 0) were killed within 12 h after birth. Animals were fed ad libitum until the start of the experiment.

### *Tissue preparation*

Diaphragms and m.flexor digitorum brevis were obtained and handled as described in section 2.2, with the modification that the diaphragm was only cut into halves in the case of 37- and 60-72-day-old rats. The weight

of m.flexor from 0-6 - day-old animals was calculated from the protein content of the incubation mixture, measured after the experiment. As the protein content of diaphragm does not alter during the suckling period (168 ± 15 (n = 36) and 165 ± 11 (n = 20) mg/g muscle for 0-6 and 9-20 -day-old rats, respectively), the value for m.flexor from 9-20 -day-old rats (131 ± 17 mg/g muscle, n = 19) was used for the calculation. Whole homogenates (5 or 10%, w/v) and mitochondria were prepared from heart, diaphragm and m.quadriceps femoralis as described in section 2.2. In the case of newborn animals the tissues from 4-6 rats of the same nest were taken together.

*Assay of palmitate oxidation and enzyme activities*

The intact muscular preparations were gently blotted on filter paper, weighed and immediately transferred to vials containing calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2) gassed with $O_2/CO_2$ (95:5), supplemented with 4 mM D,L-carnitine and 0.1 mM coenzyme A. The final incubation volume was 1 ml per 50 mg muscle with a minimum of 0.5 ml. Oxidation rates were measured with 120 µM [1-$^{14}$C]palmitate bound to dialyzed, fatty acid-free albumin in a 5:1 molar ratio. The incubations proceeded for 90 min at 37°C. Further details on the assay of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products are given in section 3.2.

Palmitate oxidation in homogenates and isolated mitochondria was measured in a medium containing 0.25 M sucrose, 30 mM KCl, 10 mM K-phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 75 mM Tris-HCl (pH 7.4), supplemented with 5 mM ATP, 1 mM $NAD^+$, 25 µM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. The final incubation volume was 0.5 ml containing 25-100 µl homogenate (1-5 mg muscle) or 25 µl mitochondrial suspension (100-200 µg protein). Incubations were carried out with 120 µM palmitate essentially as described for the intact muscular preparations (section 3.2), except that the incubation time was 15 min (mitochondria) or 30 min (homogenates).

Assays of cytochrome *c* oxidase and citrate synthase activities and of protein are described in section 2.3.

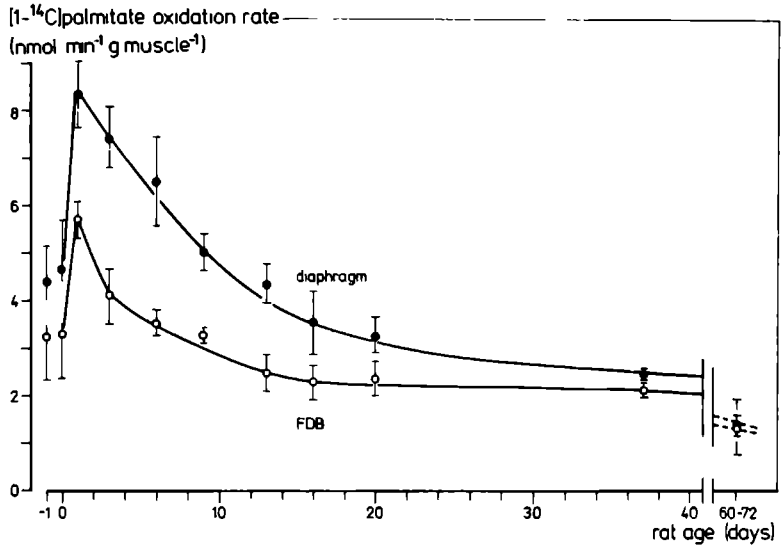



Fig. 4.1. Age dependence of palmitate oxidation by intact diaphragm or hemidiaphragm (37 and 60-72 -day-old rats) (●) and m.flexor digitorum brevis (FDB) (o). Routinely two m.flexor muscles from the same animal were incubated together. Hemidiaphragms were always taken from different animals. Values represent means ± S.D. for 4-8 experiments.

## 4.3. RESULTS

*Palmitate oxidation by intact muscular preparations*

The intactness of hemidiaphragm and m.flexor digitorum brevis was confirmed by a low leakage of soluble (cytosolic) creatine kinase during the incubation (section 3.3). Palmitate oxidation was measured routinely in the presence of carnitine and coenzyme A, as some leakage of these cofactors takes place (section 3.3).

The age dependence of the [1-$^{14}$C]palmitate oxidation rate by intact diaphragm and m.flexor is shown in Fig. 4.1. On the first day after birth the oxidation rate increases in both diaphragm and m.flexor by 178 and 173%, respectively, and falls gradually thereafter to a rate which for 60-72 -day-old rats is about 35% of that at birth. No differences were found in the oxidation rate of intact muscles from 21-day-old foetal and newborn rats. In Fig. 4.1 each value represents the mean of the oxidation

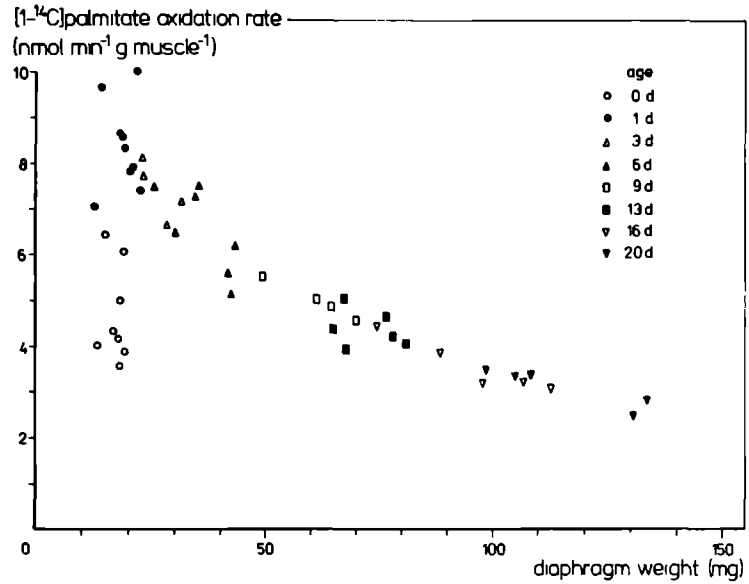



Fig. 4.2. Age dependence of palmitate oxidation by intact diaphragm. Representation of oxidation rates of individual diaphragm preparations.

rates of intact muscles from rats of the same age. The variation coefficient is rather large and amounts to 8-20% of the mean value. However, the same is true for the weights of diaphragm and m.flexor. When palmitate oxidation rates are related to muscle weight (Fig. 4.2), it appears that the individual values within one age group show a better fit to the curve. The magnitude of the standard deviation in Fig. 4.1 is thus caused partly by taking rat age as a parameter.

*Palmitate oxidation by whole homogenates*

Palmitate oxidation by whole homogenates and mitochondria proceeded at a constant rate during the incubation periods used and was proportional to the amount of tissue material. Oxidation rates did not vary much with the palmitate concentration in the range of 60-500 µM, but were maximal at 120 µM palmitate. All cofactors were present at concentrations that assured maximal oxidation rates.

In whole homogenates of all three muscles examined, heart, diaphragm and m.quadriceps, the palmitate oxidation rate increases steadily from the prenatal level, reaching a maximum at 15-20 days postnatally (Fig. 4.3).

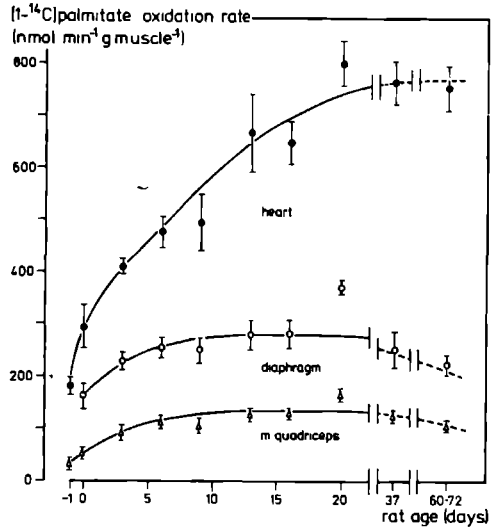



Fig. 4.3. Age dependence of palmitate oxidation by whole homogenates of heart (●), diaphragm (o) and m.quadrceps (Δ). Values represent means ± S.D. for 4-8 experiments.

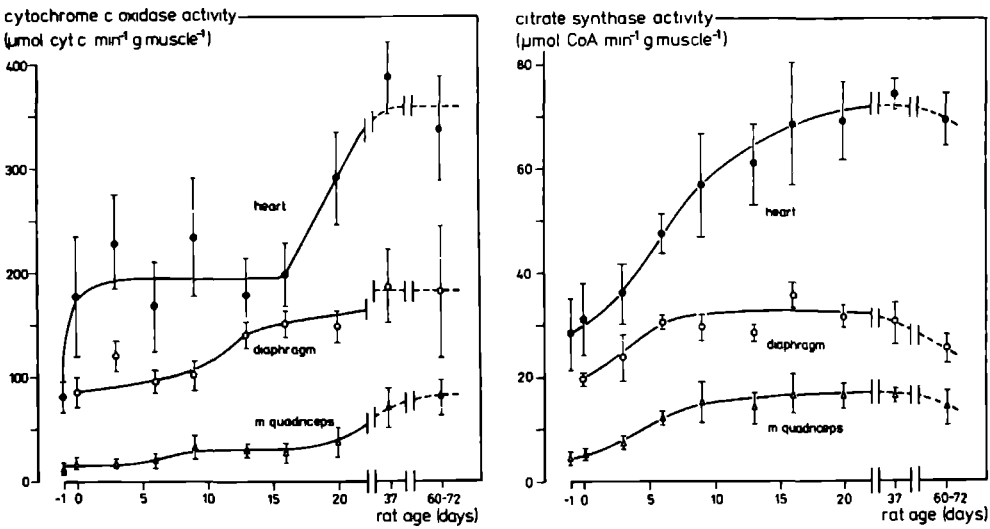



Fig. 4.4. Age dependence of the activities of cytochrome *c* oxidase (left) and citrate synthase (right), measured in whole homogenates of heart (●), diaphragm (o) and m.quadriceps (Δ). Values represent means ± S.D. for 4-14 experiments.

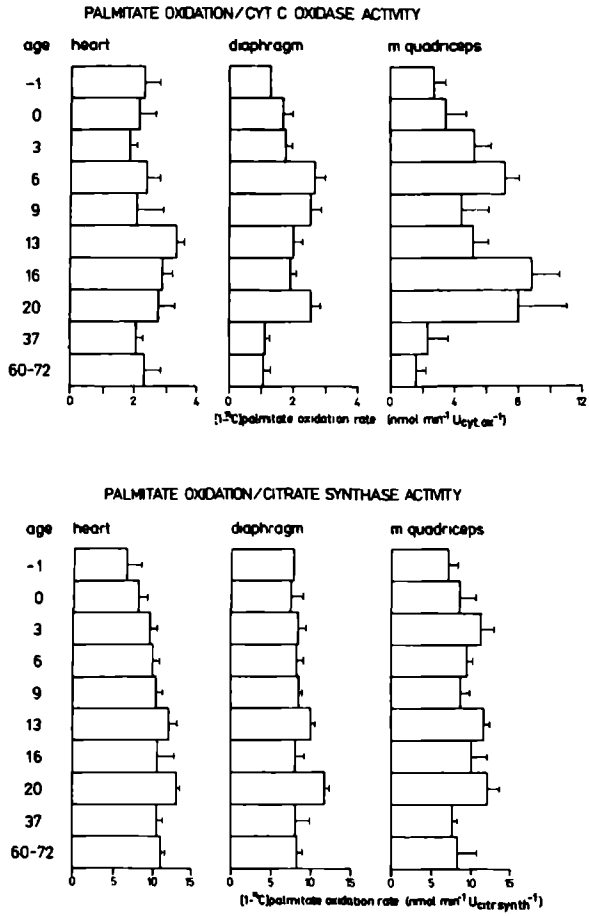



Fig. 4.5. Age dependence of palmitate oxidation relative to mitochondrial enzyme activities. Palmitate oxidation rates and the activities of cytochrome *c* oxidase and citrate synthase were measured in whole homogenates as described in section 4.2. Values represent means ± S.D. for 4-10 preparations.

This increase is especially large in heart, where the adult value is nearly three times as high as the value for newborn rats (780 *vs.* 293 nmol/min per g muscle).

*Activities of mitochondrial marker enzymes*

The activities of the mitochondrial marker enzymes cytochrome *c* oxidase and citrate synthase were measured in the whole homogenates that also had been used for the palmitate oxidation experiments. Both enzyme activi-

*Table 4.1. Comparison of tissue capacity, mitochondrial activity and mitochondrial content of muscles from newborn and adult rats.*

| | Heart | | Diaphragm | | M.quadriceps | |
|---|---|---|---|---|---|---|
| | Newborn | Adult | Newborn | Adult | Newborn | Adult |
| Tissue capacity (per g muscle) | | | | | | |
| Palmitate oxidation | 293 ± 34 | 733 ± 47 | 144 ± 26 | 213 ± 15 | 51.4 ± 8.2 | 116 ± 15 |
| Cytochrome *c* oxidase | 181 ± 60 | 341 ± 46 | 86 ± 14 | 181 ± 52 | 16.2 ± 5.8 | 84 ± 17 |
| Citrate synthase | 31.1 ± 7.8 | 71.6 ± 4.9 | 19.7 ± 1.9 | 26.6 ± 2.0 | 5.7 ± 1.1 | 13.4 ± 3.6 |
| Mitochondrial activity (per mg protein) | | | | | | |
| Palmitate oxidation | 8.79 ± 0.62 | 18.08 ± 0.46 | 4.69 ± 0.33 | 10.17 ± 0.53 | 3.12 ± 0.19 | 6.93 ± 2.04 |
| Cytochrome *c* oxidase | 5.41 ± 1.18 | 7.80 ± 1.12 | 2.19 ± 0.44 | 5.87 ± 0.81 | 1.19 ± 0.19 | 8.66 ± 2.77 |
| Citrate synthase | 0.99 ± 0.06 | 1.45 ± 0.30 | 0.55 ± 0.09 | 1.07 ± 0.07 | 0.30 ± 0.06 | 1.22 ± 0.32 |
| Mitochondrial content (mg protein/g muscle) | | | | | | |
| Palmitate oxidation | 33.3 ± 4.5 | 40.5 ± 4.5 | 30.6 ± 5.9 | 21.0 ± 1.8 | 16.5 ± 2.8 | 16.7 ± 5.3 |
| Cytochrome *c* oxidase | 33.5 ± 13.2 | 43.7 ± 8.5 | 39.3 ± 10.0 | 30.8 ± 9.8 | 13.6 ± 6.3 | 9.7 ± 3.6 |
| Citrate synthase | 31.5 ± 8.1 | 49.4 ± 10.7 | 35.8 ± 6.9 | 24.9 ± 2.5 | 18.9 ± 5.3 | 11.0 ± 4.1 |

Palmitate oxidation rates and the activities of cytochrome *c* oxidase and citrate synthase were determined in whole homogenates and isolated mitochondria as described in section 4.2. Oxidation rates and enzyme activities are expressed as nmol/min and μmol/min, respectively, per g muscle for whole homogenates and per mg protein for mitochondria. Mitochondrial protein content was calculated as tissue capacity divided by mitochondrial activity. Values are means ± S.D. for 5-10 experiments.

ties show developmental changes similar to those in the palmitate oxidation rate (Fig. 4.4). From before birth, the marker enzyme activities increase steadily to reach a maximum at 20-37 days (cytochrome *c* oxidase) and 15-20 days (citrate synthase).

Palmitate oxidation rates based on these enzyme activities are presented in Fig. 4.5. The oxidation rates thus expressed are constant during development and, moreover, are of similar magnitude for the different muscles. When expressed relative to cytochrome *c* oxidase and citrate synthase activity, the oxidation rates amount to 2-3 nmol/min per unit cytochrome *c* oxidase (except for m.quadriceps during the suckling period) and 8-12 nmol/min per unit citrate synthase. Thus, when palmitate oxidation rates are related to mitochondrial marker enzyme activities, no significant age dependence is observed, although the capacity of the mitochondrial system increases considerably during postnatal development (Figs. 4.3 and 4.4).

*Mitochondrial activity and mitochondrial content*

The increased oxidative capacity can either be the result of an increased mitochondrial activity or of an increased mitochondrial content, or a change of both. Therefore, we have compared mitochondrial activities and protein contents of heart, diaphragm and m.quadriceps from newborn (day 0) and adult rats. Mitochondrial protein content has been calculated by comparing mitochondrial parameters determined in whole homogenates and isolated mitochondria.

As parameters, we have measured palmitate oxidation rates and cytochrome *c* oxidase and citrate synthase activities in both preparations. In the case of adult rats part of the whole homogenate has always been used for the isolation of the mitochondria, in order to permit a direct comparison. The mitochondrial contents calculated by means of either of the three parameters are in reasonable agreement (Table 4.1). In cardiac muscle the increase of the tissue capacity with age is the result of a simultaneous increase in mitochondrial activity and mitochondrial content. In both skeletal muscles, diaphragm and m.quadriceps, the mitochondrial content is lower in adult than in newborn rats. Therefore, a large increase in the mitochondrial activity, particularly in m.quadriceps, is responsible for the age-related increase in the oxidative capacity in these muscles.

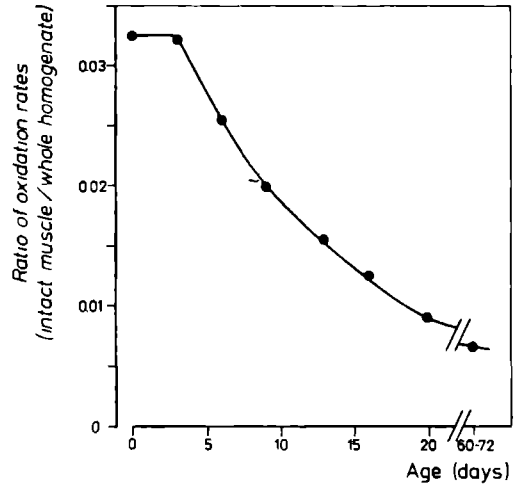



Fig. 4.6. Age-related changes in the utilization of the fatty acid oxidation capacity in diaphragm. Oxidation rates of intact diaphragm (Fig. 4.1) and of whole homogenates (Fig. 4.3) were used to calculate the ratios.

*Comparison of palmitate oxidation rates of intact muscular preparations and whole homogenates*

Oxidation rates measured in whole homogenates, expressed per g muscle, represent the best approximation of the palmitate oxidation capacity. Oxidation rates measured at the same substrate concentration in intact muscular preparations can be compared with this capacity. For diaphragm this comparison can be made over the entire age range examined. The age dependence of the ratio of oxidation rates of intact diaphragm to those in whole homogenate is shown in Fig. 4.6. The ratio is maximal at 0-3 days, but only 0.032, and decreases with age. This could indicate that the utilization of the fatty acid oxidation capacity decreases during development, but may also be related to an increase of endogenous fatty acid pools in muscle of older animals (cf. chapter 3).

## 4.4. DISCUSSION

The present study clearly demonstrates that the palmitate oxidation capacities of rat heart, diaphragm and m.quadriceps increase considerably during postnatal development. As with heart, this observation is in agreement with that of Warshaw (1972) and confirms the findings of Wittels &

Bressler (1965) that the $^{14}CO_2$ production from [1-$^{14}$C]palmitate is higher in homogenates from the adult than from the newborn rat. However, we did not find a decrease of the oxidation capacity after weaning, as was reported by Lockwood & Bailey (1970) and Bailey & Lockwood (1973). Therefore, their suggestion that changes in the metabolism of fatty acids by the developing rat heart follow changes in the fat content of the diet, does not seem likely. Association of the increase of the palmitate oxidation capacity with the postnatal appearance of lipoprotein lipase activity in both heart and skeletal muscle (Cryer & Jones, 1978; Hamosh *et al.*, 1978) is plausible. Recently, Carroll *et al.* (1983) have reported that the palmitate oxidation by homogenates of rat m.gastrocnemius increased only minimal from birth until 15 days of age and then increased more than five times until adulthood. Their oxidation rates were, however, based on only $^{14}CO_2$ production.

Some authors suggested that in rat (Wittels & Bressler, 1965; Warshaw, 1972) and bovine (Warshaw & Terry, 1970) heart the development of the fatty acid oxidation capacity would be determined by the peri- and postnatal increase of the activity of the external carnitine palmitoyltransferase. However, this hypothesis has been questioned by others, who argued that the low enzyme activities found in fetal and newborn heart could have been underestimated due to the kind of mitochondrial preparation and assay (McMillin Wood, 1975; Tomec & Hoppel, 1975).

The increase of the activities of cytochrome *c* oxidase and citrate synthase in all three muscles examined is consistent with the earlier reported postnatal rise of both enzymes in rat heart (Lang, 1965; Warshaw, 1972; Baldwin *et al.*, 1977; Vitorica *et al.*, 1981) and of citrate synthase in rat skeletal muscle (Baldwin *et al.*, 1978; Kloosterboer *et al.*, 1979) and guinea pig heart (Rolph *et al.*, 1982). However, in rabbit skeletal muscle citrate synthase activity decreases with age (Smith, 1980). For diaphragm and heart the adult values of both enzyme activities are comparable to those recently reported by Hansford *et al.* (1982) for 6-month old rats.

The origin of the age-related increase of the oxidative capacity is different for the three muscles examined. Mitochondrial activities of all muscles are elevated in the adult as compared to the newborn rat, but for m.quadriceps the difference is considerably larger than for heart or diaphragm (Table 4.1). In heart the mitochondrial content also increases with age from 31-33 (newborn) to 41-49 mg/g muscle (adult). This parallel in-

crease of both mitochondrial content and activity in heart agrees with observations of Hallman *et al.* (1972) and Kinnula & Hassinen (1977), who used cytochrome content as a mitochondrial marker. However, they reported higher values for the mitochondrial content of adult heart (80-88 mg/g muscle). On the other hand, our value is identical to those calculated by others (Abu-Erreish & Sanadi, 1978; Idell-Wenger *et al.*, 1978; Feuvray & Plouet, 1981), who used cytochrome *c* oxidase and citrate synthase activity as mitochondrial markers. Also in guinea pig heart both the mitochondrial content and activity were found to increase during fetal and postnatal development (Rolph *et al.*, 1982).

The mitochondrial content of both diaphragm and m.quadriceps decreases with age (Table 4.1). M.gastrocnemius from 7-day-old rats showed a mitochondrial content of 16 mg/g muscle (Kloosterboer *et al.*, 1979), which corresponds to our results with m.quadriceps. Recently, Davies *et al.* (1981), using several enzyme activities and content of cytochromes as parameters, reported an average value of 19 mg/g muscle for adult skeletal muscle. The age-dependent decrease can be attributed in part to dispersion or dilution of mitochondria by myofibrillar protein (Burleigh, 1974). Our finding that the mitochondrial content and fatty acid oxidation capacity are higher in diaphragm than in m.quadriceps agrees with results of histochemical studies. Adult diaphragm mainly consists of slowly contracting oxidative fibers (Close, 1972), while adult m.quadriceps is a rather mixed-type muscle (Baldwin *et al.*, 1972). Although the functions of diaphragm and m.quadriceps differ substantially, the pattern of metabolic development is similar for the two muscles.

In muscle a distinction has been made between subsarcolemmal and intermyofibrillar mitochondria. Several investigators reported that these two types of mitochondria differ metabolically in that the intermyofibrillar mitochondria of both cardiac (Palmer *et al.*, 1977) and skeletal muscle (Krieger *et al.*, 1980; Brady & Tandler, 1981) show higher state 3 respiration rates and enzyme activities. However, recently, Matlib *et al.* (1981) have presented evidence that these differences can be created during isolation and assay. They have not found differences in cytochrome content and enzyme activities between the two mitochondrial classes. In our studies the mitochondrial preparations isolated after hand homogenization of adult rat muscle predominantly contain subsarcolemmal mitochondria. In newborn rat muscles the two mitochondrial classes cannot be distinguished.

Therefore, if there are metabolic differences between the two mitochondrial populations, this could affect our conclusions.

The rapid increase of the palmitate oxidation rate in both intact diaphragm and m.flexor immediately after birth may be related to the enhancement of the functional capacity of the muscles during development. The subsequent decrease may be related to three factors: an impeded transport of substrate in muscle of older animals, an increase of endogenous fatty acid pools, and changes in the regulation of fatty acid oxidation in the intact muscle cell during postnatal development. The pyruvate oxidation rate of rat diaphragm shows a similar decrease with age (Zuurveld, J.G.E.M. and Veerkamp, J.H., unpublished observations).

For all developmental stages examined, the palmitate oxidation rates are considerably lower in intact muscular preparations than in homogenates. Possible explanations for this observation were already given in section 3.4.

## 4.5. SUMMARY

The palmitate oxidation rate has been measured in intact diaphragm and m.flexor digitorum brevis and in whole homogenates of heart, diaphragm and m.quadriceps femoralis of developing rats between late fetal life and maturity. Activities of the mitochondrial enzymes cytochrome *c* oxidase and citrate synthase have also been determined.

Immediately after birth the palmitate oxidation rate increases markedly in both intact diaphragm and m.flexor and falls gradually after day 1 to adult values which are about 35% of those at birth.

The oxidation capacities of diaphragm and m.quadriceps, but especially of heart, increase steadily during development, starting before birth and reaching adult values at 15-20 days postnatally. The activities of the mitochondrial enzymes show a similar developmental pattern. In heart the increase of oxidative capacity is the result of an increase of both mitochondrial content and mitochondrial activity. In diaphragm and m.quadriceps the mitochondrial contents decrease with age, so the increase of their oxidative capacities is due to a large rise of the mitochondrial activity.

# CHAPTER 5

# MITOCHONDRIAL AND PEROXISOMAL PALMITATE OXIDATION*

## 5.1. INTRODUCTION

In earlier studies from this laboratory it has been observed that postnuclear fractions and mitochondria from muscle showed a higher production of $^{14}CO_2$ and $^{14}C$-labeled perchloric acid-soluble products from [1-$^{14}$C]palmitate than from [U-$^{14}$C]palmitate (Van Hinsbergh *et al.*, 1978a,b, 1979, 1980). It was suggested that accumulation of intermediates during the β-oxidation may cause this phenomenon. Accumulation of β-oxidation intermediates as coenzyme A esters was demonstrated during state 3 oxidation of palmitate by rat liver mitochondria (Stanley & Tubbs, 1974, 1975). Carnitine esters of these intermediates were observed with liver mitochondria after oxidation of palmitate with carnitine and fluorocitrate in the absence of ADP (Lopes-Cardozo *et al.*, 1978).

Peroxisomal β-oxidation is essentially not complete in contrast to mitochondrial oxidation (Lazarow, 1978). The former system has a maximal activity towards acyl-CoA esters of 12-18 carbon atoms and little towards $C_6$ and $C_4$ acyl-CoA esters (Lazarow, 1978; Osumi & Hashimoto, 1978; Hryb & Hogg, 1979). Therefore, the observed incomplete degradation of palmitate molecules (Van Hinsbergh *et al.*, 1978a,b, 1979, 1980) may be due to peroxisomal release of intermediates, which are not further oxidized within the mitochondria.

In this study we compared the palmitate oxidation capacity and the extent of incomplete palmitate degradation in various rat tissues. Assays were performed in intact muscles, whole homogenates, 600 x *g* supernatants and mitochondrial preparations, which may contain different proportions of mitochondria and peroxisomes. The peroxisomal contribution to the pal-

*Taken and adapted from Veerkamp *et al.* (1983) and Glatz & Veerkamp (1982).

mitate oxidation was established in homogenates of rat skeletal muscle by use of various inhibitors of mitochondrial oxidation.

Since skeletal muscle contains a relatively high carnitine concentration (Brooks & McIntosh, 1975; Van Hinsbergh *et al.*, 1980), the accumulation of carnitine esters may occur under physiological conditions. Therefore, we investigated whether carnitine and inhibition of carnitine palmitoyltransferase influenced the dependence of the oxidation rate on the label position of [$^{14}$C]palmitate in muscle mitochondria and homogenates. The identity of the intermediates was studied by the analysis of the acid-soluble and acid-insoluble fractions after incubation of muscle and liver homogenates and mitochondria.

## 5.2. MATERIALS AND METHODS

Male random-bred albino Wistar rats were used. The rats weighed either 90-120 g (intact muscular preparations) or 180-240 g (cell-free systems), and were starved for 18 h before the start of the experiment. Biopsies from human m.pectoralis were obtained and handled as described in section 2.1. The preparation of the intact muscles and of the various cell-free systems is described in section 2.2.

### *Assays*

Palmitate oxidation rates of intact muscular preparations were measured as described in section 3.2. With cell-free systems, palmitate oxidation rates were measured in a total volume of 0.5 ml containing 25 mM sucrose, 75 mM Tris-HCl (pH 7.4), 10 mM $K_2HPO_4$, 5 mM $MgCl_2$ and 1 mM EDTA, supplemented with 1 mM $NAD^+$, 5 mM ATP, 25 μM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. 120 μM [1-$^{14}$C]palmitate bound to albumin in a 5:1 molar ratio was used as substrate, unless otherwise indicated. A volume of 50-100 μl homogenate or 600 × *g* supernatant or of 25 μl mitochondrial suspension (100-200 μg protein) was added. Incubation was carried out for 15 min (mitochondria) or 30 min at 37°C. Oxidation was stopped by adding 0.2 ml of 3 M perchloric acid and followed by a second incubation for 90 min at 4°C. $^{14}CO_2$ produced was bound to 0.2 ml ethanolamine/ethyleneglycol (1:2, v/v). Thereafter the acid incubation mixture was centrifuged in a Burkard Koolspin microcentrifuge, and 200 μl supernatant was assayed for radioactivity.

Radioactivity of $^{14}CO_2$ in the trapping solution was determined after addition of 10 ml toluene/methanol (2:1, v/v) containing 0.4% Omnifluor. Radioactivity of the acid-soluble metabolites was assayed after addition of 10 ml Aquasol.

Protein and citrate synthase activity were assayed as described in section 2.3.

Peroxisomal oxidation of palmitate or palmitoyl-CoA was assayed in a modification of the system described by Lazarow (1981b). Production of $^{14}C$-labeled acid-soluble products was measured after 10 min incubation at 37°C of 100 µl rat liver or muscle homogenate in a total volume of 0.5 ml. The medium contained 50 mM sucrose, 0.01% Triton X-100, 0.1 mM coenzyme A, 0.2 mM $NAD^+$, 10 µM FAD, 5 mM ATP, 1 mM dithiothreitol, 73 µM antimycin A, 10 µM rotenon and 45 mM Tris-HCl (pH 7.4). Palmitate or palmitoyl-CoA and albumin concentrations are indicated in the Table.

*Identification of the acid-insoluble products*

For assay of $^{14}C$-labeled fatty acids the total acid-insoluble fraction was washed with water and hydrolyzed for 2 h at 80°C in methanol/water (1:1, v/v) with 1 M KOH. After evaporation of methanol and acidification, fatty acids were extracted with pentane and separated on silanized silica gel plates in acetic acid/water (3:1, v/v). The presence of $^{14}C$-labeled hydroxy fatty acids was investigated by thin-layer chromatography of the fatty acid fraction before and after methylation (Fleming & Haigre, 1973). Chloroform (Hull *et al.*, 1972) and hexane/diethylether/acetic acid (30:70:1, by vol) were used as solvent systems on silica gel G.

The $^{14}C$-labeled compounds were located by autoradiography and radioactivity of the individual compounds was determined by liquid scintillation counting.

## 5.3. RESULTS

All assays were performed under conditions which were optimal with respect to time and concentrations of palmitate, malate and of tissue material (chapter 3). The intactness of mitochondria in the homogenates was checked by assay of released citrate synthase activity. Without sonication the relative activity of this mitochondrial matrix enzyme amounted to 16 ± 2 and 17 ± 3% (means ± S.D. for 4 and 6 experiments) in the 20,000 × *g*

*Table 5.1. Proportional contribution of $^{14}CO_2$ production to the [1-$^{14}$C]palmitate oxidation rate by cell-free preparations of rat tissues.*

| Tissue | Homogenate | Mitochondria |
|---|---|---|
| M. quadriceps | 6 ± 3 | 13 ± 4 |
| Diaphragm | 12 ± 5 | 10 ± 2 |
| Heart | 18 ± 3 | 16 ± 2 |
| Liver | 2 ± 2 | 1 ± 1 |
| Kidney | 8 ± 6 | 25 ± 5 |
| Brain | 7 ± 2 | 2 ± 2 |
| Lung | 7 ± 3 | - |

Values are given in % of the total activity of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble compounds and are means ± S.D. of 3-22 experiments.

supernatant prepared from homogenates of rat heart and m.quadriceps, respectively. The centrifugation procedure did not influence the release, since the same percentages were found in 600 × *g* supernatants. The mitochondrial preparations showed good values of respiratory control index and ADP/O ratio with 1 mM 2-oxoglutarate as substrate (Van Hinsbergh *et al.*, 1978a, 1980).

In the following text, [$^{14}$C]palmitate oxidation will always refer to its conversion into the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products. The $^{14}CO_2$ production from [1-$^{14}$C]palmitate represented 1-25 and 50-60% of this sum in rat cell-free systems and intact muscles, respectively (Tables 5.1 and 5.2). With [16-$^{14}$C]- and [U-$^{14}$C]palmitate the $^{14}CO_2$ production amounted to only 1-2% of the total oxidation rate in heart, muscle and lung homogenates, was not detected with other cell-free preparations, but represented about 30% with the intact muscles (Table 5.2).

Table 5.3 (page 76) presents the oxidation rates of palmitate measured in mitochondrial preparations, 600 × *g* supernatants and whole homogenates from various rat tissues. The capacity for palmitate oxidation is similar and highest in homogenates from heart and liver. Kidney has a higher capacity than diaphragm and m.quadriceps. Lung and brain have a low palmitate oxidation capacity. The activities of the post-nuclear fraction (600 × *g* supernatant) do not give quite the same picture, since homogenization liberates different proportions of the number of mitochondria

*Table 5.2. Ratio of oxidation rates of [1-$^{14}$C]- and [16-$^{14}$C]palmitate by intact preparations of human and rat muscle and the contribution of $^{14}CO_2$ production.*

| Muscle | | Ratio of oxidation rates | Contribution of $^{14}CO_2$ production (%) | |
|---|---|---|---|---|
| | | | 1-$^{14}$C | 16-$^{14}$C |
| Human | m.pectoralis fibers | 1.00 ± 0.02 | 31 ± 5 | 11 ± 2 |
| Rat | hemidiaphragm | 1.01 ± 0.05 | 51 ± 2 | 30 ± 2 |
| | m.soleus | 1.01 ± 0.03 | 53 ± 3 | 29 ± 1 |
| | m.flexor digitorum brevis | 0.98 ± 0.11 | 58 ± 3 | 30 ± 2 |

Ratios of oxidation rates were determined in paired experiments with 120 µM $^{14}$C-labeled palmitate bound to albumin (molar ratio 5:1). In case of m.pectoralis teased fibers from the same muscle were used for each experiment. The proportional contributions of $^{14}CO_2$ production to the total oxidation rate with |1-$^{14}$C|- and |16-$^{14}$C|palmitate are given in % of the sum of the production of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble compounds. All values represent means ± S.D. of 4 experiments.

present in the various tissues and since the tissue content of protein varies. The mitochondria from heart show the highest palmitate oxidation rate, those from skeletal muscle, liver and kidney have an intermediate activity and those from brain a very low activity.

In all cell-free systems the oxidation rate was higher with [1-$^{14}$C]- than with [16-$^{14}$C]palmitate or [U-$^{14}$C]palmitate (Table 5.3). The ratio of oxidation rates was always larger for [1-$^{14}$C]- and [16-$^{14}$C]- than for [1-$^{14}$C]- and [U-$^{14}$C]palmitate. The highest ratios were observed with the post-nuclear fractions. With intact preparations of rat and also of human skeletal muscle, however, we did not observe a difference between the oxidation rates with [1-$^{14}$C]- and [16-$^{14}$C]palmitate (Table 5.2), despite the marked difference of the relative contributions of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products to the total oxidation rate. Identical oxidation rates were also found with 1 mM [1-$^{14}$C]- and [16-$^{14}$C]palmitate (data not shown).

*Table 5.3. Palmitate oxidation by cell-free preparations of rat tissues.*

| Tissue | Preparation | [1-$^{14}$C]Palmitate oxidation rate | Ratio of oxidation rates | |
|---|---|---|---|---|
| | | | [1-$^{14}$C]-/ [16-$^{14}$C]-palmitate | [1-$^{14}$C]-/ [U-$^{14}$C]-palmitate |
| M.quadriceps | mitochondria | 6.90 ± 1.51 | 1.60 ± 0.22 | 1.38 ± 0.19 |
| | 600 x *g* supernatant | 0.49 ± 0.10 | 2.13 ± 0.36 | 1.32 ± 0.23 |
| | whole homogenate | 113 ± 17 | 1.38 ± 0.14 | 1.15 ± 0.10 |
| Diaphragm | 600 x *g* supernatant | 0.82 ± 0.10 | 1.55 ± 0.11 | 1.34 ± 0.15 |
| | whole homogenate | 192 ± 37 | 1.39 ± 0.17 | 1.30 ± 0.18 |
| Heart | mitochondria | 16.71 ± 3.09 | - | 1.24 ± 0.11 |
| | 600 x *g* supernatant | 1.48 ± 0.26 | 1.54 ± 0.13 | 1.20 ± 0.13 |
| | whole homogenate | 760 ± 104 | 1.18 ± 0.07 | 1.14 ± 0.06 |
| Liver | mitochondria | 6.35 ± 1.76 | 1.47 ± 0.19 | 1.32 ± 0.16 |
| | 600 x *g* supernatant | 1.11 ± 0.24 | 1.91 ± 0.27 | 1.54 ± 0.14 |
| | whole homogenate | 659 ± 95 | 1.54 ± 0.21 | 1.66 ± 0.24 |
| Kidney | mitochondria | 3.94 ± 0.93 | 1.46 ± 0.11 | 1.16 ± 0.11 |
| | 600 x *g* supernatant | 0.94 ± 0.15 | 1.79 ± 0.27 | 1.45 ± 0.17 |
| | whole homogenate | 303 ± 40 | 1.61 ± 0.21 | 1.55 ± 0.22 |
| Brian | mitochondria | 0.59 ± 0.09 | 2.42 ± 0.07 | 1.62 ± 0.12 |
| | 600 x *g* supernatant | 0.16 ± 0.03 | 2.92 ± 0.66 | 1.69 ± 0.13 |
| | whole homogenate | 16.7 ± 2.9 | 2.22 ± 0.25 | 1.91 ± 0.22 |
| Lung | whole homogenate | 4.5 ± 3.3 | 1.56 ± 0.19 | - |

Values are means ± S.D. of 4-38 preparations. Rates are given in nmol/min per mg protein for mitochondrial fractions and 600 x *g* supernatants, and in nmol/min per g tissue (wet weight) for whole homogenates. 120 μM $^{14}$C-labeled palmitate bound to albumin (molar ratio 5:1) was used as substrate.

We investigated on m.quadriceps and liver which factors may cause or influence the ratio of oxidation rates found with cell-free systems for palmitate labeled in different carbon atoms. Disruption of rat muscle mitochondria by one cycle of freezing and thawing decreased the oxidation rate by 50 ± 5%, mean ± S.D., n = 3), but the ratio did not change significantly (1.84 ± 0.14 *vs.* 1.75 ± 0.04 for disrupted and intact mitochondria, respectively). More cycles of freezing and thawing or addition of Triton X-100 decreased the oxidation rate further and increased the ratio to 2-4. Addition of disrupted mitochondria or a 20,000 × *g* supernatant from the homogenate to intact mitochondria or to homogenates did not influence the ratio.

*Influence of various parameters on palmitate oxidation by mitochondrial preparations*

The omission of L-carnitine decreased markedly the oxidation rate of m.quadriceps mitochondria, but did not influence significantly the ratio (Table 5.4; page 78). Increase of the carnitine concentration above 0.5 mM had no marked effect. The same was observed with rat liver mitochondria (data not shown). The concentration of mitochondrial protein and/or of palmitate and the palmitate:albumin molar ratio (at 120 µM palmitate) did not significantly affect the ratio. The apparent $K_m$ for L-carnitine was 80-100 µM with muscle mitochondria. The palmitate oxidation rate is nearly maximal at 0.5 mM L-carnitine.

Addition of KCN or antimycin A to muscle mitochondria eliminated all $^{14}CO_2$ production and decreased markedly less the production of radioactive acid-soluble products from [1-$^{14}$C]- than from [16-$^{14}$C]palmitate, so that the ratio of the oxidation rates becomes much larger (Table 5.4). The effect of addition of both antimycin and rotenon was not significantly different from that of antimycin alone (data not shown).

For comparison with omission of carnitine, the effect of malonyl-CoA, a specific inhibitor of carnitine palmitoyltransferase (McGarry *et al.*, 1978a), was also studied. Malonyl-CoA decreased the oxidation rate of [1-$^{14}$C]palmitate by about 50%, but less that of [16-$^{14}$C]palmitate, so that the rates became about equal. Production of $^{14}CO_2$ and formation of $^{14}$C-labeled acid-soluble products from [1-$^{14}$C]palmitate were inhibited to a similar extent. Earlier (Veerkamp & Van Moerkerk, 1982) we established that the effect of malonyl-CoA on rat skeletal muscle mitochondria was maximal at 50 µM and not dependent on the nutritional state.

*Table 5.4. Influence of various parameters on palmitate oxidation by mitochondria of rat m.quadriceps.*

| Parameter changed | Relative oxidation rate | Ratio of oxidation rates [1-$^{14}$C]- / [16-$^{14}$C]palmitate |
|---|---|---|
| Carnitine concentration | | |
| Control (0.5 mM) | 100 | 1.74 ± 0.25 |
| 0 mM | 7 ± 3 [a] | 1.85 ± 0.43 |
| 0.1 | 74 ± 9 [a] | 1.79 ± 0.27 |
| 0.2 | 87 ± 7 | 1.87 ± 0.18 |
| 1.0 | 113 ± 8 [b] | 1.79 ± 0.41 |
| 2.0 | 120 ± 6 [a] | 2.21 ± 0.27 [b] |
| Protein concentration | | |
| 100 µg/ml | 94 ± 9 | 1.56 ± 0.16 |
| 200 | 100 | 1.50 ± 0.16 |
| 500 | 110 ± 6 | 1.74 ± 0.31 |
| Palmitate concentration | | |
| 30 µM | 98 ± 18 | 1.47 ± 0.33 |
| 60 | 110 ± 17 | 1.82 ± 0.28 |
| 120 | 100 | 1.78 ± 0.34 |
| Palmitate:albumin molar ratio | | |
| 1:1 | 71 ± 10 | 1.78 ± 0.44 |
| 3:1 | 89 ± 12 | 1.69 ± 0.20 |
| 5:1 | 100 | 1.51 ± 0.06 |
| Addition | | |
| none | 100 | 1.60 ± 0.23 |
| 20 mM KCN | 28 ± 5 [a] | 3.33 ± 1.18 [a] |
| 36 µM antimycin A | 33 ± 11 [a] | 4.40 ± 1.38 [a] |
| 50 µM malonyl-CoA | 48 ± 11 [a] | 1.15 ± 0.08 [a] |
| 100 µM malonyl-CoA | 48 ± 5 [a] | 1.05 ± 0.06 [a] |

Values are means ± S.D. of 5-12 experiments. Relative oxidation rates of [1-$^{14}$C]palmitate are given in % of the corresponding control values, set at 100 (0.5 mM L-carnitine, 120 µM palmitate bound to albumin in a 5:1 molar ratio and about 200 µg protein per assay). Statistical significance was analyzed with Student's t-test for paired data: different from the control with [a] $P < 0.001$, [b] $P < 0.01$.

*Table 5.5. Influence of various parameters on palmitate oxidation by homogenates of rat m.quadriceps.*

| Omission or addition | Relative oxidation rate | Ratio of oxidation rates [1-$^{14}$C]- / [16-$^{14}$C]palmitate |
|---|---|---|
| None | 100 | 1.41 ± 0.18 |
| - $NAD^+$ | 94 ± 3 | - |
| - L-carnitine | 19 ± 12 [a] | 1.37 ± 0.20 |
| 10 mM KCN | 41 ± 8 [a] | 1.88 ± 0.46 [b] |
| 20 mM KCN | 24 ± 5 [a] | 2.72 ± 0.87 [a] |
| 4 µM antimycin A | 19 ± 2 [a] | 3.66 ± 0.44 [a] |
| 36 µM antimycin A | 19 ± 5 [a] | 3.71 ± 1.38 [a] |
| 50 µM malonyl-CoA | 53 ± 5 [a] | 1.16 ± 0.06 [a] |
| 100 µM malonyl-CoA | 47 ± 9 [a] | 1.11 ± 0.05 [a] |

Values are means ± S.D. of 10-15 experiments. Relative oxidation rates of [1-$^{14}$C]palmitate are given in % of the corresponding control value, set at 100. Statistical significance for paired data is indicated in Table 5.4.

*Influence of some parameters on palmitate oxidation by homogenates*

Omission of carnitine decreased the oxidation rate less with homogenates (Table 5.5) than with mitochondria (Table 5.4), since endogenous carnitine (about 0.02 mM) is present in homogenates. Palmitate oxidation was hardly dependent on the carnitine concentration between 0.1 and 2 mM (data not shown), as also found with mitochondria (Table 5.4). Omission of carnitine had no effect on the ratio. Omission of $NAD^+$ had only a slight effect on the oxidation rate, which also demonstrates the intactness of the mitochondria. Malonyl-CoA decreased the oxidation rate of [1-$^{14}$C]palmitate by about 50%, similarly as with mitochondria. The ratio decreased also significantly in the presence of malonyl-CoA (Table 5.5). To investigate the cause of this latter decrease the oxidation of [1-$^{14}$C]tetradecanoate was studied. Malonyl-CoA inhibited the oxidation of this acid (134 ± 21 nmol/min per g) by only 35 ± 5% (mean ± S.D., 7 experiments).

No $^{14}CO_2$ production was observed from [$^{14}$C]palmitate in the presence of KCN or antimycin A. The ratio of the oxidation rates of [1-$^{14}$C]- and [16-$^{14}$C]palmitate increased markedly in the presence of both inhibitors (Table 5.5). Also the ratios with [1-$^{14}$C]- and [3-$^{14}$C]- or [U-$^{14}$C]palmi-

*Table 5.6. Peroxisomal fatty acid oxidation by homogenates of rat liver and m.quadriceps.*

| Substrate | Concentrations (μM) | Liver | M.quadriceps |
|---|---|---|---|
| [1-$^{14}$C]Palmitate (albumin) | 120 (24) | 30.6 ± 9.8 | 2.0 ± 0.5 |
| [1-$^{14}$C]Palmitoyl-CoA (albumin) | 20 (1) | 24.4 ± 5.6 | 9.6 ± 1.0 |
| | 40 (1) | 37.7 ± 8.4 | 15.3 ± 2.5 |
| | 60 (1) | 46.6 ± 5.3 | 17.9 ± 0.5 |
| | 80 (1) | 51.4 ± 8.9 | 18.6 ± 0.6 |

Production of $^{14}$C-labeled acid-soluble products was assayed in a medium containing 0.01% Triton X-100, 0.1 mM coenzyme A, 0.2 mM $NAD^+$, 5 mM ATP, 73 μM antimycin A and 10 μM rotenon. Incubation was carried out for 15 min at 37°C. Oxidation rates are given in nmol/min per g tissue as means ± S.D. of 3 or 4 experiments. Albumin concentrations are given within parentheses.

tate were considerably higher in the presence of KCN or antimycin A (data not shown). With the 600 x *g* supernatant from muscle omission of carnitine and addition of 10 mM KCN or 4 μM antimycin A decreased the [1-$^{14}$C]palmitate oxidation rate to 16 ± 1, 29 ± 11 and 14 ± 8%, respectively (means ± S.D. of 3-5 experiments). Addition of antimycin A to muscle homogenates did not affect the low oxidation rates of [1-$^{14}$C]palmitate in the absence of carnitine, but decreased still more the [16-$^{14}$C]palmitate oxidation. The ratio rose from 1.49 ± 0.11 to 4.32 ± 0.69 (means ± S.D. of 4 experiments).

*Peroxisomal palmitate oxidation*

The occurrence of a cyanide- and antimycin-insensitive palmitate oxidation in homogenates and mitochondrial fractions even with omission of L-carnitine, suggested the existence of a peroxisomal fatty acid oxidation system in muscle. In order to establish this, the assay according to the method of Lazarow (1981b) was applied in the presence of antimycin and rotenon (Table 5.6). With liver homogenates palmitate and palmitoyl-CoA were good substrates. With muscle homogenates palmitoyl-CoA had a 10-fold higher oxidation rate, which was comparable with the rate observed in the usual assay of palmitate oxidation in the presence of antimycin and rotenon. We also measured the palmitoyl-CoA -dependent $H_2O_2$ production indi-

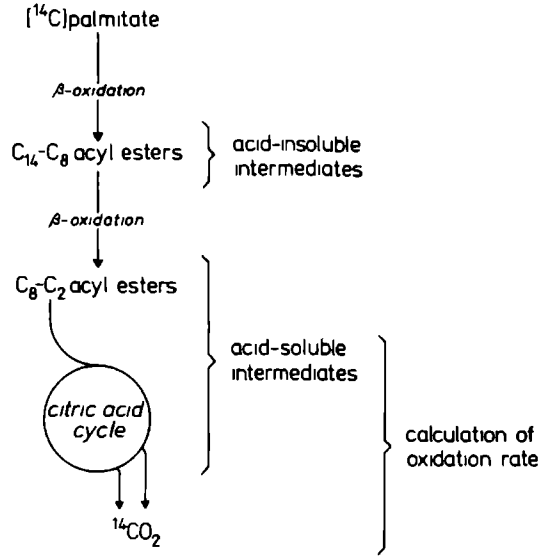



Fig. 5.1. Stages of palmitate oxidation. Oxidation rates are calculated from the production of $^{14}CO_2$ and $^{14}C$-labeled perchloric acid-soluble products. The latter consist of short-chain acyl-CoA and acyl-carnitine esters and citric acid cycle intermediates.

rectly with the oxidation of methanol to formaldehyde (Mannaerts *et al.*, 1979). The rate of formaldehyde production was constant up to 30 min with muscle homogenates but varied markedly in separate experiments (7.7 ± 4.6 nmol/min per g, mean ± S.D. of 4 experiments). The rate of formaldehyde production is, however, not equivalent with the $H_2O_2$ production and always lower than the palmitate oxidation rate (Mannaerts *et al.*, 1979).

*Analysis of fatty acid oxidation intermediates*

Upon incubation of homogenates of m.quadriceps or of liver with [$^{14}C$]palmitate labeled at different positions, the acid-soluble fraction (Fig. 5.1) was found to contain, besides citric acid cycle intermediates (Van Hinsbergh *et al.*, 1978a,b, 1979), always only [$^{14}C$]acetylcarnitine or [$^{14}C$]acetic acid (Veerkamp *et al.*, 1983).

The lipid composition of the perchloric acid-insoluble fraction (Fig. 5.1) was investigated to detect to what extent [$^{14}C$]palmitate was incorporated or converted to other compounds. After incubation of rat muscle mitochondria with [$^{14}C$]palmitate free fatty acids represented 95-98% of the

*Table 5.7. $^{14}C$-labeled oxidation products of [16-$^{14}C$]- and [1-$^{14}C$]palmitate in homogenates and mitochondrial fractions of rat m.quadriceps and liver.*

| Substrate | Product(s) | Presence of antimycin | Muscle | | Liver | |
|---|---|---|---|---|---|---|
| | | | Homogenate (6) | Mitochondria (3) | Homogenate (3) | Mitochondria (3) |
| [16-$^{14}C$]Palmitate | Dodecanoic acid | - | 92 ± 60 | 10 ± 4 | 1005 ± 120 | 3 ± 1 |
| | | + | 74 ± 42 | 11 ± 6 | 870 ± 210 | n.d. |
| | Tetradecanoic acid | - | 221 ± 104 | 28 ± 6 | 2835 ± 525 | 17 ± 3 |
| | | + | 191 ± 75 | 36 ± 10 | 2385 ± 395 | n.d. |
| | $CO_2$ and acid-soluble products | - | 1380 ± 120 | 86 ± 20 | 6735 ± 135 | 73 ± 18 |
| | | + | 84 ± 24 | 11 ± 3 | 465 ± 120 | n.d. |
| [1-$^{14}C$]Palmitate | $CO_2$ and acid-soluble products | - | 1695 ± 240 | 118 ± 15 | 10965 ± 780 | 94 ± 14 |
| | | + | 330 ± 75 | 61 ± 18 | 3010 ± 345 | n.d. |

Homogenates and mitochondrial fractions were incubated for 15 min with 120 μM [$^{14}C$]palmitate. The concentration of antimycin A was 36 μM. Amounts of $^{14}C$-labeled dodecanoic and tetradecanoic acid were determined by thin-layer chromatography after alkaline hydrolysis of the acid-insoluble fraction. Values are means ± S.D. for the number of experiments given within parentheses and are expressed in nmol/g tissue for homogenates and in nmol/mg protein for mitochondria. n.d., not determined.

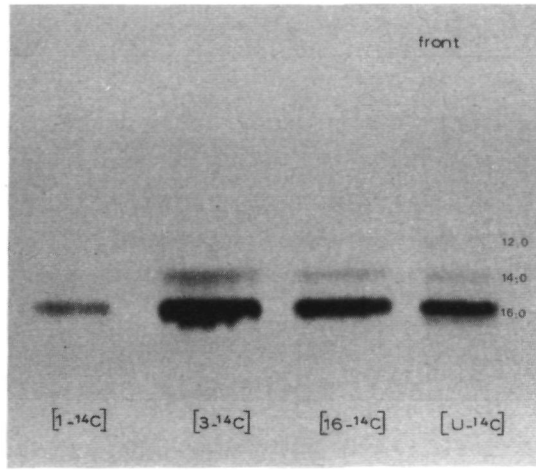



Fig. 5.2. Radioautogram of the $^{14}C$-labeled fatty acids isolated from the acid-insoluble fractions of incubations of rat m.quadriceps homogenate with [$^{14}C$]palmitate labeled in different positions. The silanized silica gel plate was developed in acetic acid/water (75:25, v/v).

$^{14}C$-radioactivity of the neutral lipids.

For a proper identification and quantitation of intermediates, homogenates and mitochondria from rat liver and m.quadriceps were incubated with palmitate labeled in different positions. The fatty acids were isolated after alkaline hydrolysis of total isolated lipids and separated by thin-layer chromatography. Fig. 5.2 shows a representative autoradiogram for experiments with muscle homogenates. Incubation with [3-$^{14}C$]palmitate resulted in the appearance of $^{14}C$-labeled tetradecanoic acid, whereas incubations with[ U-$^{14}C$]- and [16-$^{14}C$]palmitate gave also labeled dodecanoic and decanoic acids. After incubation with or without antimycin A no $^{14}C$-labeled hydroxy acids could be detected, neither their derived methyl esters after methylation of the lipid fraction.

The absolute amounts of the chain-shortened fatty acids vary markedly in the different systems and also between the experiments in one system (Table 5.7). This is in accordance with the variability of the ratio. The amount of decanoic acid was in all cases less than 10% of that of dodecanoic acid. The values for tetradecanoic acid were similar after incubation with [3-$^{14}C$]- or [16-$^{14}C$]palmitate. The amount of tetradecanoic acid was

about 2-3-fold of that of dodecanoic acid in all systems investigated. Only with liver mitochondria much more of the former acid was present (Table 5.7).

Antimycin A did not significantly influence the amounts of $^{14}C$-labeled chain-shortened fatty acids in contrast to its marked decrease of the oxidation of [16-$^{14}C$]palmitate. These observations explain the considerable increase of the ratio of oxidation rates of [1-$^{14}C$]- and [16-$^{14}C$]palmitate in the presence of antimycin A (Tables 5.4 and 5.5). The amounts of chain-shortened fatty acids recovered can nearly account in all systems, with and without antimycin, for the differences in the oxidation rates of [1-$^{14}C$]- and [16-$^{14}C$]palmitate (Table 5.7).

## 5.4. DISCUSSION

This chapter gives approximate values for the capacity of palmitate oxidation in homogenates and in mitochondria of various rat tissues. The assay of the sum of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products is a more sensitive and accurate method for determination of [$^{14}C$]fatty acid oxidation in these systems than is measurement of $^{14}CO_2$ alone (cf. chapter 3), although the latter method is still applied (e.g. Sumbilla *et al.*, 1981; Okano & Shimojo, 1982; Barakat *et al.*, 1982, 1983; Shumate *et al.*, 1982). The acid-soluble intermediates represent the predominant products in mitochondria and homogenates of all tissues investigated, as was earlier reported for rat liver (McGarry *et al.*, 1977; Christiansen, 1978; Kawamura & Kishimoto, 1981), human and rat muscles (Van Hinsbergh *et al.*, 1978b, 1979, 1980; Shumate & Choksi, 1981) and rat brain ( Kawamura & Kishimoto, 1981). In perfused, isolated rat heart (Oram *et al.*, 1973) $^{14}CO_2$ appeared to be the main product of [$^{14}C$]palmitate oxidation, but at incubation of intact skeletal muscle preparations (chapter 3) it also accounts for only a part of the palmitate oxidation products.

Oxidation rates of palmitate in liver homogenates and mitochondria are comparable to those observed by other investigators (Lopes-Cardozo & Van den Bergh, 1972; McGarry *et al.*, 1977; 1978c; Mannaerts *et al.*, 1979; Kawamura & Kishimoto, 1981). Our values with rat heart and kidney homogenates were twice as high as previously reported (McGarry *et al.*, 1978c; cf. chapter 7), presumably due to a higher palmitate:albumin molar ratio in our assays. Capacities of palmitate oxidation in homogenates of other rat tis-

sues were either not determined earlier or measured with $^{14}CO_2$ production (Wittels & Bressler, 1965; Baldwin *et al.*, 1972; Engel & Angelini, 1973; Warshaw & Terry, 1976; Willner *et al.*, 1979) or with oxygen consumption (Warshaw, 1972; Hooker & Baldwin, 1979). Our values in rat heart and skeletal muscle homogenates are comparable with the latter (Hooker & Baldwin, 1979). The capacity of rat heart homogenates agrees quite well with the rate of β-oxidation (Oram *et al.*, 1973; Pearce *et al.*, 1979) and oxygen consumption (Taegtmeyer *et al.*, 1980) in the perfused working rat heart. The postnuclear fractions and mitochondria from rat brain and especially liver had a much higher activity than found at a low palmitate concentration (Vignais *et al.*, 1958; Kawamura & Kishimoto, 1981).

The concentration of L-carnitine necessary for maximal palmitate oxidation appeared to be lower than the normal concentration in rat skeletal muscle (Brooks & McIntosh, 1975; Van Hinsbergh *et al.*, 1980). The apparent $K_m$ of about 100 μM L-carnitine in muscle mitochondria is comparable with the value of 190 μM for rat liver mitochondria (Van Tol, 1974). With mitochondria from rabbit skeletal muscle the oxidation rate was 40% lower at 0.2 mM than at 1.5 mM carnitine (Pande, 1971).

The differences in the oxidation rates observed with differently labeled palmitate in cell-free preparations of various rat tissues and also of human muscles (Veerkamp *et al.*, 1983) can be caused by various mechanisms. Futile cycling by a combination of shortening and elongation of the fatty acid chain (Whereat, 1971) does not seem probable. The formation of [$^{14}$C]-octadecanoic acid from [$^{14}$C]palmitate and of [$^{14}$C]dodecanoic acid from [3-$^{14}$C]palmitate was not observed. A second possibility is that intermediates escape from the multienzyme complex of β-oxidation, as was found in conditions of state 4 respiration and inhibited citric acid cycle (Lopes-Cardozo *et al.*, 1978) or respiratory chain (Stanley & Tubbs, 1974, 1975). Under normal conditions of state 3 palmitate oxidation, carnitine may transport intermediates out of the mitochondrion. Long-chain acyl-carnitine hydrolase and acyl-CoA hydrolase (Lin & Kako, 1975; Berge & Døssland, 1979) can contribute to the formation of free chain-shortened fatty acids. These acids were present in both the free fatty acid and the acyl-carnitine fraction, since both fractions showed an inverse ratio in comparison to the oxidation products (Veerkamp *et al.*, 1983). A release of β-oxidation intermediates from the mitochondria does, however, not appear the main cause for the increase of the chain-shortened fatty acids. The carnitine concentra-

tion did not influence the ratio of the oxidation rates in muscle mitochondria and homogenates. Release of carnitine esters from damaged mitochondria cannot be the cause, since such preparations showed very low oxidation rates, their addition to homogenates had no effect on the ratio, and the percentage of damaged mitochondria in the homogenates was low.

Since KCN and antimycin markedly decreased the oxidation rate and increased the ratio, but did not affect the nature and amount of formed intermediates, it appears probable that the chain-shortened fatty acids result from peroxisomal oxidation (Fig. 5.3). The comparable oxidation rates in the presence and absence of carnitine and the more direct estimations according to the procedure of Lazarow (1981b) and of formaldehyde production indicate also that peroxisomal oxidation takes place in rat and human muscle.

The presence of cyanide-insensitive fatty acid oxidation was found in liver and kidney of many species (Lazarow & De Duve, 1976; Bronfman *et al.*, 1979; Tolbert, 1981), in rat brain (Kawamura & Kishimoto, 1981) and also in human and rat muscle (Shumate & Chokski, 1981). Fatty acid oxidation by isolated peroxisomes was only established with rat liver (Lazarow, 1978), but acyl-CoA oxidase activity ($H_2O_2$-producing) was also found in heart and skeletal muscle of guinea pigs (Small *et al.*, 1980) and in mouse heart (Connock & Perry, 1983). Cytochemical methods demonstrated the presence of peroxisomes in liver, striated muscles and other tissues (Hand, 1974; Christie & Stoward, 1979; Tolbert, 1981). Very recently, Norseth *et al.*(1982, 1983) reported the isolation and hydrodynamic parameters of microperoxisomes from rat heart, and observed the stimulation of microperoxisomal β-oxidation in heart by a high-fat diet.

The production of chain-shortened fatty acids by peroxisomes appears probable, since peroxisomal oxidation of palmitoyl-CoA is incomplete and involves at the most 5 β-oxidation cycles (Lazarow, 1978). This maximum was never reached with liver peroxisomal fractions (Thomas *et al.*, 1980) and products of the first 2-3 β-oxidation cycles predominate (Osmundsen *et al.*, 1980a).The rate of appearance of acid-soluble radioactivity from [1-$^{14}$C]-palmitoyl-CoA was one order of magnitude greater than from [U-$^{14}$C]palmitoyl-CoA (Osmundsen *et al.*, 1980a).With oleate oxidation shortened acyl-carnitines were detected in hepatocytes (Osmundsen *et al.*, 1980b). We detected only saturated chain-shortened fatty acids after incubation of rat muscle and liver homogenates and mitochondria with palmitate, also in the presence

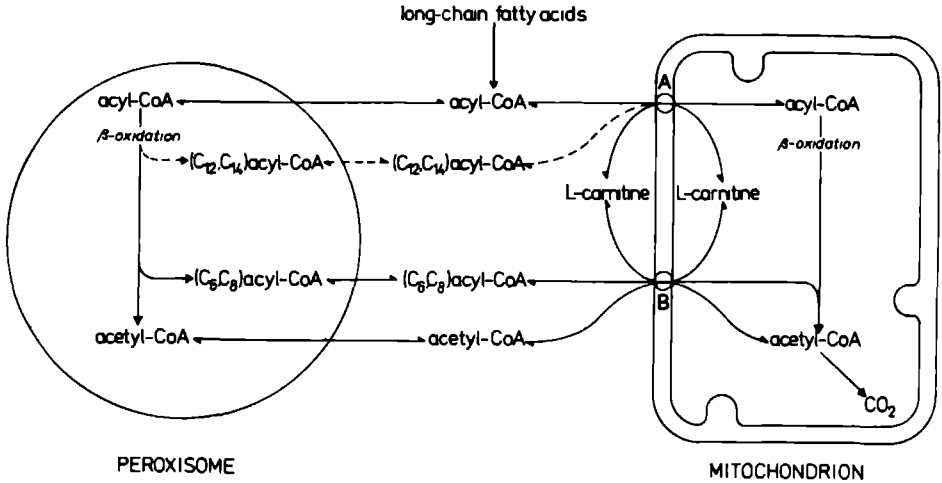



Fig. 5.3. Schematic presentation of long-chain fatty acid degradation. The interrelationship between peroxisomes and mitochondria is given. $C_{12}$ and $C_{14}$ intermediates may escape from the peroxisomal oxidation pathway and accumulate in the medium. A, carnitine long-chain acyltransferase; B, carnitine short-chain acyltransferase.

of antimycin A. Terminal respiratory-chain inhibition by cyanide in rabbit heart mitochondria (Moore *et al.*, 1982) caused no accumulation of hydroxy fatty acids in contrast to inhibition with rotenon in mitochondria from rat liver (Stanley & Tubbs, 1975; Bremer & Wojtczak, 1972) and heart (Christophersen & Christiansen, 1975) and rabbit heart (Moore *et al.*, 1982). The marked decrease of the ratio by malonyl-CoA is presumably caused by a more complete degradation of the peroxisomal chain-shortened fatty acids, since oxidation of tetradecanoic acid is less inhibited than that of palmitate.

The variations in the ratios in the different conditions can be explained by the extent of interaction of peroxisomal and mitochondrial palmitate oxidation (Fig. 5.3). Important in this respect may be the degree of intactness of the peroxisomes and mitochondria (Thomas *et al.*, 1980), their relative amounts in the various fractions, and the extent to which these organelles cooperate to degrade the long-chain fatty acids to $CO_2$ and acid-soluble products in the cell-free systems, which are 20-100-fold diluted in comparison to the intact cell. Homogenates generally show lower ratios and thus a more complete degradation of the palmitate molecule than the postnuclear fractions. With intact muscle preparations and heart slices

(section 7.3) there is no difference between the oxidation rates with [1-$^{14}$C]- and [16-$^{14}$C]palmitate. In hepatocytes (Christiansen, 1978) and in well-oxygenated perfused rat heart (Markwell *et al.*, 1976) the ratio was also about one. With rat liver mitochondria an equal degradation of [1-$^{14}$C]- and [U-$^{14}$C]palmitate was found only at a very high concentration of mitochondrial protein (Lopes-Cardozo *et al.*, 1978). At protein concentrations from 250 µg to 12 mg per ml, however, accumulation of labeled intermediates was observed (Stanley & Tubbs, 1975). The intermediates found in incubations with mitochondria can originate from peroxisomal oxidation, since about 50% of total peroxisomal marker enzyme activities are present in conventional liver mitochondrial preparations (Osmundsen, 1981).

In conclusion, the results of our experiments indicate that the differences between the oxidation rates observed with palmitate labeled in different positions are mainly due to variations of the extent of degradation of peroxisomal oxidation products by mitochondria in the various cell-free systems.

## 5.5. SUMMARY

The palmitate oxidation capacity has been determined in whole homogenates, postnuclear fractions and mitochondrial fractions of various muscles, liver, kidney, brain and lung, and in intact preparations of various skeletal muscles of the rat.

The oxidation rate (calculated from the sum of the production of $^{14}CO_2$ and $^{14}$C-labeled perchloric acid-soluble intermediates) was with [1-$^{14}$C]palmitate > [U-$^{14}$C]palmitate > [16-$^{14}$C]palmitate in all cell-free systems. With the intact muscular preparations, however, the oxidation rate was not dependent on the position of the $^{14}$C-label in the palmitate molecule.

Oxidation capacities were highest in rat heart and liver, intermediate in kidney, diaphragm and m.quadriceps, and low in brain and lung. Omission of L-carnitine and addition of malonyl-CoA, KCN or antimycin A decreased the oxidation rates in whole homogenates and mitochondrial fractions. Antimycin A or KCN increased and malonyl-CoA decreased the ratio between the oxidation rates with [1-$^{14}$C]- and [16-$^{14}$C]palmitate. Omission of L-carnitine had no significant effect on the ratio.

$^{14}$C-labeled dodecanoic and tetradecanoic acids were identified in

homogenates and mitochondrial fractions of m.quadriceps and liver as acid-insoluble intermediates of [16-$^{14}$C]palmitate oxidation in the presence and absence of antimycin A. Their amounts recovered can account for the differences in oxidation rates found with [1-$^{14}$C]- and [16-$^{14}$C]palmitate in the cell-free systems. The occurrence of peroxisomal fatty acid oxidation was demonstrated in muscle homogenates.

The incomplete palmitate oxidation in cell-free systems appears to be mainly caused by an inadequate mitochondrial degradation of peroxisomal oxidation products.

# CHAPTER 6

# EFFECT OF CLOFIBRATE FEEDING ON PALMITATE OXIDATION IN RAT LIVER AND MUSCLE*

## 6.1. INTRODUCTION

Clofibrate (ethyl 2-(4-chlorophenoxy)2-methylpropanoate) is an effective hypolipidemic and hypocholesterolemic agent in man and rat (Thorp & Waring, 1962; Havel & Kane, 1973). Chronic administration of this drug to rodents increases the weight of the liver and its content of mitochondria (Kurup *et al.*, 1970; Lipsky & Pedersen, 1982) and peroxisomes (reviewed by Reddy *et al.*, 1982). The marked increase of the peroxisomal β-oxidation of fatty acids (Lazarow & De Duve, 1976; Lazarow, 1977) suggested a prominent role of this system in the effects of clofibrate. Both mitochondrial and peroxisomal oxidation of palmitate appear, however, to increase markedly in hepatocytes and liver homogenates of clofibrate-fed rats (Mannaerts *et al.*, 1978, 1979; Christiansen *et al.*, 1978; Pande & Parvin, 1980; Bremer *et al.*, 1981). Perfused livers from such rats showed an increased oleate oxidation, but also an increased fatty acid synthesis, measured with $^3H_2O$ incorporation (Laker & Mayes, 1979). The drug induces, especially, a series of enzymes and proteins involved in hepatic fatty acid metabolism (Bremer *et al.*, 1981). Oxidation of pyruvate and succinate were unaltered in liver homogenates (Mannaerts *et al.*, 1978), like that of acetate and various citric acid cycle intermediates in isolated liver mitochondria from clofibrate-fed rats (Cederbaum *et al.*, 1976; Rasheed *et al.*, 1980). The oxidation of palmitoylcarnitine by isolated liver mitochondria was reported to decrease (Cederbaum *et al.*, 1976), to increase (Mackerer, 1977; Pande & Parvin, 1980) and not to change (Christiansen *et al.*, 1978), presumably due to variations in conditions of assay or in mitochondrial preparations.

Clofibrate appeared also to affect occasionally skeletal muscle in

*Adapted from Glatz *et al.* (1983a).

treated patients (Langer & Levy, 1968; Bridgman *et al.*, 1972). This effect was partly related to an impaired fatty acid and glucose oxidation by Paul & Adibi (1979). These investigators used, however, $^{14}CO_2$ production as a measure for fatty acid oxidation. Since $^{14}CO_2$ forms only a small fraction of the oxidation products from [$^{14}$C]palmitate in homogenates of liver and muscle (Mannaerts *et al.*, 1978; Van Hinsbergh *et al.*, 1978a,b; Pande & Parvin, 1980), we reevaluated their data by determining palmitate oxidation from the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products. Simultaneously, we determined the rate of antimycin-insensitive oxidation in muscle to explore possible peroxisomal contribution. These activities were compared with the activities of two mitochondrial marker enzymes, citrate synthase (EC 4.1.3.7) and cytochrome *c* oxidase (EC 1.9.3.1).

## 6.2. MATERIALS AND METHODS

Male albino Wistar rats of 200-240 g were used. The composition of the diet and the administration of clofibrate were as described in section 2.1. Whole homogenates of liver (5%, w/v) and m.quadriceps femoralis (10%, w/v) were prepared as described in section 2.2.

The palmitate oxidation rate was measured in a total volume of 0.5 ml medium, containing 50 µl whole homogenate and 25 mM sucrose, 75 mM Tris-HCl (pH 7.4), 10 mM $K_2HPO_4$, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM $NAD^+$, 5 mM ATP, 25 µM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. The concentration of [1-$^{14}$C]- or [16-$^{14}$C]palmitate (bound to albumin in a molar ratio of 5:1) was 120 µM. In parallel incubations 36 µM antimycin A was added to inhibit mitochondrial oxidation. After 30 min incubation at 37°C the incubation was stopped by addition of 0.2 ml of 3 M perchloric acid. Radioactivity of trapped $CO_2$ and of acid-soluble products was measured as described in section 3.2.

The assay procedures for cytochrome *c* oxidase and citrate synthase activities are described in section 2.3.

All activities were calculated per g tissue (wet weight). Statistical significance was analyzed with Student's *t*-test for unpaired data.

## 6.3. RESULTS

All assays were established to be proportional with time of incubation and amount of tissue material. The concentrations of substrates, coenzymes and cofactors were optimal (Van Hinsbergh *et al.*, 1978a,b). Clofibrate treatment had no effect on rat weights.

Both clofibrate feeding and short starvation increased very significantly the total palmitate oxidation capacity per g wet weight of rat liver homogenates (Table 6.1). Since starvation caused a decrease of the liver weight by 23% (from 10.2 ± 0.4 to 7.9 ± 0.5 g, n = 5), the total oxidation capacity expressed per whole liver increased by about 50%. Based on cytochrome *c* oxidase and citrate synthase activity the palmitate oxidation rates increased in liver homogenates at 18 h starvation by 38 and 41%, respectively.

*Table 6.1. Effect of clofibrate treatment and short starvation on total and antimycin-insensitive palmitate oxidation in liver and muscle homogenates.*

| Tissue | Condition | Palmitate oxidation | | |
|---|---|---|---|---|
| | | Total (nmol/min per g) | Antimycin-insensitive (nmol/min per g) | (% of total) |
| Liver | Fed (5) | 366 ± 28 | 142 ± 17 | 39 ± 5 |
| | Clofibrate-fed (10) | 978 ± 255 [a] | 390 ± 74 [a] | 37 ± 5 |
| | Starved 18 h (8) | 713 ± 92 [a] | 165 ± 16 | 23 ± 2 [a] |
| Muscle | Fed (10) | 130 ± 24 | 15 ± 2 | 11 ± 2 |
| | Clofibrate-fed (10) | 125 ± 27 | 15 ± 2 | 11 ± 1 |
| | Starved 18 h (11) | 115 ± 19 | 16 ± 5 | 14 ± 3 |

Values are means ± S.D. of the number of animals given within parentheses. Total palmitate oxidation was determined from $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products formed with [1-$^{14}$C]palmitate as substrate. Antimycin-insensitive palmitate oxidation was calculated as the difference in oxidation rates of [1-$^{14}$C]- and [16-$^{14}$C]palmitate in the presence of 36 μM antimycin A. [a] *P versus* fed animals < 0.001.

*Table 6.2. Effect of clofibrate treatment and short starvation on activities of citrate synthase and cytochrome c oxidase in liver and muscle homogenates.*

| Tissue | Condition | Citrate synthase | Cytochrome *c* oxidase |
|---|---|---|---|
| Liver | Fed | 11.7 ± 1.2 (10) | 286 ± 41 (7) |
| | Clofibrate-fed | 13.8 ± 1.8 (10) | 355 ± 38 (10) |
| | Starved 18 h | 15.4 ± 2.0 [a] (41) | 317 ± 98 (30) |
| Muscle | Fed | 11.9 ± 2.6 (12) | 81 ± 12 (9) |
| | Clofibrate-fed | 8.5 ± 1.0 [a] (10) | 67 ± 7 [b] (9) |
| | Starved 18 h | 13.5 ± 2.6 (56) | 68 ± 20 (41) |

Values are given in units per g wet weight tissue and represent means ± S.D. of the number of animals given within parentheses.
*P versus* fed animals: [a] < 0.001; [b] < 0.01

$^{14}CO_2$ production was only about 6, 4 and 1% of the total oxidation rate of [1-$^{14}$C]palmitate with liver homogenates from fed, clofibrate-fed and 18 h-starved rats, respectively. The rate of $^{14}CO_2$ production was, therefore, also higher in liver homogenates with clofibrate-fed rats than with fed animals, but lower with 18 h-starved animals.

Antimycin completely inhibited $^{14}CO_2$ production from [1-$^{14}$C]- and [16-$^{14}$C]palmitate and decreased the acid-soluble products from [16-$^{14}$C]-palmitate to about 10% (cf. Table 5.5). The latter values were applied to correct the rate of [1-$^{14}$C]palmitate oxidation in the presence of antimycin for mitochondrial contribution. Starvation increased only the antimycin-sensitive, or mitochondrial, oxidation, while clofibrate treatment increased also the antymicin-insensitive oxidation in liver homogenates (Table 6.1).

In muscle homogenates $^{14}CO_2$ was in all three conditions about 4-6% of the sum of $^{14}CO_2$ and radioactive acid-soluble products. $^{14}CO_2$ production rates were lower with clofibrate-fed rats than with fed animals (4.1 ± 1.0 (n = 10) *versus* 7.5 ± 2.6 (n = 9) nmol/min per g wet weight). The latter observations are similar to those of Paul & Adibi (1979). When palmitate oxidation rates were, however, calculated from the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products, no differences were observed between the muscle

homogenates from rats in all three conditions (Table 6.1). The contribution of the antimycin-insensitive palmitate oxidation was also not influenced in muscle homogenates by the condition of the rat.

In liver homogenates clofibrate treatment did not change the activities of citrate synthase or of cytochrome *c* oxidase (Table 6.2). The only significant effect of clofibrate on muscle was a decrease of the activities of these mitochondrial marker enzymes. Short starvation only increased the citrate synthase activity in liver.

The same homogenates were also used to measure the oxidation rates of pyruvate, leucine and the branched-chain 2-oxo-acids derived from leucine (4-methyl-2-oxopentanoate) and valine (3-methyl-2-oxobutanoate). With these substrates significant alterations in the clofibrate-treated and the starved rats were also predominantly observed in the liver. The results of these investigations are described elsewhere (Glatz *et al.*, 1983c).

## 6.4. DISCUSSION

A simultaneous estimation of the effect of clofibrate feeding on the palmitate oxidation capacity in liver and muscle homogenates was not earlier reported. Together with palmitate oxidation, both mitochondrial and peroxisomal (Lazarow & De Duve, 1976; Lazarow, 1977; Mannaerts *et al.*, 1978, 1979; Bremer *et al.*, 1981) (Table 6.1), also pyruvate and 4-methyl-2-oxopentanoate oxidation increased in liver homogenates, but 3-methyl-2-oxobutanoate oxidation and the activities of citrate synthase and cytochrome *c* oxidase were not changed (Glatz *et al.*, 1983c). The latter observations establish that not all mitochondrial activities increase, as was earlier also found with isolated liver mitochondria (Cederbaum *et al.*, 1976; Rasheed *et al.*, 1980). The unchanged cytochrome *c* oxidase activity was also reported by Mannaerts *et al.* (1978) in combination with an increased glutamate dehydrogenase activity. Lazarow & De Duve (1976) and Lazarow (1977) noted a variability in the effects of clofibrate on cytochrome *c* oxidase activity. The lower concentration of cytochrome $aa_3$ in liver mitochondria from clofibrate-treated rats (Reddy *et al.*, 1982) is in accordance with the simultaneous increase of mitochondrial protein (Reddy *et al.*, 1982) and the unchanged cytochrome *c* oxidase activity.

Our observations on the effect of clofibrate feeding on fatty acid oxidation in liver are in accordance with earlier results (Christiansen *et*

*al.*, 1978; Mannaerts *et al.*, 1978, 1979; Pande & Parvin, 1980; Bremer *et al.*, 1981). With our assay of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products we did not find a decrease of palmitate oxidation in muscle homogenates as deduced by Paul & Adibi (1979) from $^{14}CO_2$ production. No effect of clofibrate administration was also observed on the oxygen consumption of muscle mitochondria with palmitoyl-L-carnitine as substrate (Cederbaum *et al.*, 1976). In perfused rat heart and isolated rat heart mitochondria (Norseth, 1980) clofibrate feeding appeared to increase palmitate oxidation by about 25 and 48%, respectively.

Starvation for only 15-24 h increases fatty acid oxidation in liver homogenates, when it is based on wet weight (McGarry *et al.*, 1977; Table 6.1). In contrast to McGarry *et al.* (1977) we found also an increase of the of oxidation capacity for whole liver. In hepatocytes this process is also increased (Ontko, 1972; McGarry *et al.*, 1978b). No increase was observed with liver mitochondria (Ontko & Johns, 1980; Cook *et al.*, 1980; McGarry & Foster, 1981), muscle homogenates (Tables 3.2 and 6.1) and mitochondria (Veerkamp & Van Moerkerk, 1982) or intact muscle preparations (Table 3.2). The mitochondrial content of liver from starved rats shows only a small increase, when based on citrate synthase (Table 6.2), in contrast to the reported 60% increase, based on glutamate dehydrogenase activity (McGarry *et al.*, 1977). In muscle homogenates from clofibrate-treated rats we observed only a slight decrease of the activities of citrate synthase and cytochrome *c* oxidase, but these enzymes are not rate-limiting in oxidative metabolism.

The present results, combined with those reported elsewhere (Glatz *et al.*, 1983c) do not sustain the conclusions of Paul & Adibi (1979, 1980) that fatty acid and glucose oxidation are impaired and leucine and branched-chain 2-oxo acid oxidation are increased in muscle of clofibrate-fed rats. The effects of clofibrate feeding on liver metabolism can, however, indirectly influence muscle metabolism *in vivo*. Clofibric acid (2-(4-chlorophenoxy)2-methylpropanoate), which is present in a high concentration in the plasma during clofibrate treatment (Laker & Mayes, 1979) may also influence the uptake of metabolites, their oxidative metabolism in muscle and/or the membrane characteristics of muscle, and induce in this way myopathic phenomena. Clofibric acid may displace other organic ligands from plasma albumin into tissues, as suggested already by Thorp in 1963 (Thorp, 1963). It interferes with the binding of fatty acids (Spector & Santos,1973).

An interesting observation is that in liver clofibrate treatment also markedly increases the concentrations of long-chain acyl-CoA and of total coenzyme A (Ball *et al.*, 1979; Skrede & Halvorsen, 1979; Voltti & Hassinen, 1980), and the concentration of carnitine and the activities of carnitine acyltransferases (Pande & Parvin, 1980; Kerner & Bieber, 1982). In addition, the hepatic content of the fatty acid-binding protein (FABP), which binds acyl-CoA esters (Mishkin & Turcotte, 1974), increases after clofibrate treatment (Fleischner *et al.*, 1975; Renaud *et al.*, 1978; Kawashima *et al.*, 1982). Since the palmitate oxidation capacity rises simultaneously (Table 6.1), these findings strongly suggest that FABP is involved in the regulation of fatty acid oxidation (cf. chapter 10).

## 6.5. SUMMARY

Total and antimycin-insensitive palmitate oxidation rates and the activities of two mitochondrial marker enzymes (citrate synthase and cytochrome *c* oxidase) have been assayed in liver and muscle homogenates of fed, clofibrate-fed and 18 h-starved rats.

Significant alterations in the clofibrate-treated and the starved rats are predominantly observed in the liver. Clofibrate feeding increases antimycin-insensitive (peroxisomal) and antimycin-sensitive (mitochondrial) palmitate oxidation in liver. In muscle only the activities of citrate synthase and cytochrome *c* oxidase decrease slightly. Short starvation only increases antimycin-sensitive palmitate oxidation in liver.

Our results suggest that myopathic phenomena observed after chronic clofibrate administration are not related to changes in the capacity of oxidative metabolism of muscle.

# CHAPTER 7

# PALMITATE OXIDATION IN HUMAN AND RAT HEART: COMPARISON OF CELL-FREE AND CELLULAR SYSTEMS*

## 7.1. INTRODUCTION

Free fatty acids constitute the main energy source of the heart under aerobic conditions (Neely & Morgan, 1974). For the *in vitro* study of myocardial fatty acid metabolism several tissue preparations can be used. Distinction should first be made between preparations that contain intact cells (cellular systems) and those in which the cells have been disrupted (cell-free systems). Studies with cellular systems have long been hampered by the lack of a suitable procedure for the isolation of viable and calcium-tolerant heart cells. Now that several of such procedures have been described (Vahouny *et al.*, 1979; Altschuld *et al.*, 1980; Frangakis *et al.*, 1980; Haworth *et al.*, 1980; Dow *et al.*, 1981; reviewed by Farmer *et al.*, 1983), these cells have also become an important model system for biochemical studies.

In all investigations reported on fatty acid oxidation in myocytes and in most studies in other preparations oxidation rates were based on the measurement of $^{14}CO_2$ production. Since $^{14}CO_2$ forms only a fraction of the oxidation products from [$^{14}$C]fatty acids in cell-free (Van Hinsbergh *et al.*, 1978a, 1979) as well as in cellular (chapter 3) systems of heart and skeletal muscle and other tissues, we use a more accurate and sensitive procedure in which the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products is measured. Previously (Van Hinsbergh *et al.*, 1979) the palmitate oxidation rates in the postnuclear fraction (600 x *g* supernatant) and isolated mitochondria of human and rat heart have been determined. The capacity of this oxidation process can, however, more adequately be studied in whole homogenates than in these systems, since homogenization and differential cen-

* Adapted from Glatz *et al.* (1983c).

trifugation procedures may select certain populations of mitochondria (Palmer *et al.*, 1977). The aim of the present study is to compare the metabolic characteristics of cell-free and cellular systems of heart and also to compare palmitate oxidation rates of human and rat heart. In this chapter we report palmitate oxidation rates and activities of mitochondrial marker enzymes measured in mitochondria, whole homogenates and slices of human and rat heart and in rat cardiac myocytes.

In order to further examine the differences between human and rat heart we calculated their mitochondrial contents from the data of oxidation rates and activities of cytochrome *c* oxidase and citrate synthase in whole homogenates and mitochondria. Since carnitine is not synthesized in the heart (Rebouche, 1982) we have studied the effect of its addition to cellular systems on the fatty acid oxidation. Furthermore, special attention has been paid to the significance of endogenous lipolysis and the contribution of damaged cells to the fatty acid oxidation in the cellular systems. The observed differences between human and rat heart and the characteristic properties of the various systems are discussed.

## 7.2. MATERIALS AND METHODS

### *Preparation of cell-free and cellular systems*

Biopsies (200-500 mg) of the right auricle of adult human heart were obtained and handled as described in section 2.1. Rat heart was obtained from male random-bred albino Wistar rats (180-240 g), which had been fasted for 18 h. The preparation of heart slices and whole homogenates and the isolation of mitochondria are described in section 2.2.

Rat cardiac myocytes were isolated by perfusion of the heart with collagenase essentially according to the procedure of Altschuld *et al.* (1980) with some modifications. The heart was perfused *in vitro* through the aorta at 37°C with a nominally calcium-free Krebs-Ringer bicarbonate buffer, composed of 119 mM NaCl, 5 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$ and 25 mM $NaHCO_3$ (pH 7.2), gassed with $O_2/CO_2$ (95:5) and supplemented with 11 mM glucose. After a washout period of 5-10 min a recirculating perfusion was started with 25 ml of the same buffer supplemented with 0.1% (w/v) collagenase. Perfusion was continued for 45-60 min. The softened heart then was placed in 10 ml perfusion medium without collagenase but containing 1% (w/v) fatty acid-free albumin, minced with scissors and incubated for an

additional 10 min at 30°C. During incubation the cells were gently dispersed with a pipet. The resultant dispersion was sieved through 300-µm nylon mesh and centrifuged for 1 min at 50 × *g*. After discarding the supernatant, the cells were washed once. Finally, they were suspended in calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2), gassed with $O_2/CO_2$ (95:5). The cells were counted with a hemocytometer and their viability judged by determination of the percentage of myocytes excluding trypan blue. In some experiments viable and non-viable cells were separated by isopycnic centrifugation on a Percoll gradient for 3 min at 400 × *g* according to Maisch *et al.* (1981), with the modification that an isotonic Percoll solution was used.

*Assay of palmitate oxidation*

With homogenates and isolated mitochondria, the fatty acid oxidation rate was measured radiochemically at 37°C in 20-ml incubation vials in a medium containing 25 mM sucrose, 30 mM KCl, 10 mM K-phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 75 mM Tris-HCl (pH 7.4) supplemented with 5 mM ATP, 1 mM $NAD^+$, 25 µM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. The final incubation volume was 0.5 ml, containing 25-50 µl homogenate (1.3 - 2.5 mg muscle) or 25 µl mitochondrial suspension (100-200 µg protein). The vials were equipped with polypropylene center wells and sealed with rubber stoppers. After 5 min preincubation at 37°C the reaction was started by the addition of 120 µM [1-$^{14}$C]palmitate bound to fatty acid-free albumin (molar ratio 5:1), unless otherwise indicated. The oxidation proceeded for 30 min (homogenates) or 15 min (mitochondria) at 37°C and was terminated by the addition of 0.2 ml 3 M perchloric acid to the medium, followed by a second incubation for 75 min at 4°C. The released $^{14}CO_2$ was trapped in 0.3 ml ethanolamine/ethylene glycol (1:2, v/v) injected into the center well. Thereafter, the acid incubation mixture was centrifuged in a Burkard Koolspin microcentrifuge (1 min, 10,000 × *g*), and an aliquot of the supernatant was assayed for radioactivity by liquid scintillation counting in 10 ml of Aqualuma. Radioactivity of $^{14}CO_2$ was measured in 10 ml toluene/methanol (2:1, v/v) containing 0.4% Omnifluor. Palmitate oxidation rates were calculated from the production of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products.

With mitochondria palmitate oxidation was additionally determined polarographically in the same medium at 37°C with a Clark oxygen electrode

as described by Van Hinsbergh *et al.* (1978a).

Palmitate oxidation by heart slices and isolated heart cells was measured in calcium-free Krebs-Ringer bicarbonate buffer (pH 7.2), gassed with $O_2/CO_2$ (95:5). The final incubation volume was 2.5 ml, containing 40-70 mg muscle (slices) or 0.6 - 1.5 mg protein (myocytes). Incubations were carried out essentially as described above, except that the incubation time was 90 min, 0.5 ml 3 M perchloric acid was used and the second incubation lasted for 18 h at 4°C.

*Assay of lipolysis*

The rate of endogenous lipolysis in heart slices was determined from the release of glycerol. Heart slices were incubated for 40 min under the same conditions as described for the assay of palmitate oxidation. Aliquots of the medium were taken at the end of each incubation. Glycerol was determined as described in section 2.3.

*Other assays*

Assays of protein and of the activities of cytochrome *c* oxidase, citrate synthase and creatine kinase are described in section 2.3.

*Statistics*

Statistical significance was analyzed by Student's *t*-test.

## 7.3. RESULTS

*Palmitate oxidation by cell-free systems*

Functional intactness of the isolated mitochondria from both human and rat heart was confirmed by good respiratory control index values (4 or higher) and ADP/O ratios (2.2-2.5), when measured with an oxygen polarograph and 1 mM 2-oxoglutarate or L-glutamate as substrate. Possible injury of the mitochondria during homogenization was assayed by the release of citrate synthase activity into the medium. Without sonication, the relative activity of this mitochondrial matrix enzyme, determined in the 20,000 x *g* supernatant prepared from rat heart homogenates, was only 16 ± 2% (mean ± S.D. of 4 preparations). These results show that the cell-free preparations contain functionally and structurally intact mitochondria. With our isolation procedure the yield of mitochondria ranged from 12 to 20% (both human

*Table 7.1. Proportional contribution of $^{14}CO_2$ to [1-$^{14}$C]palmitate oxidation rate.*

| Species and system | | | Contribution of $^{14}CO_2$ |
|---|---|---|---|
| Human heart | mitochondria | (5) | 4 ± 1 |
| | homogenate | (15) | 4 ± 2 |
| | slices | (7) | 33 ± 6 |
| Rat heart | mitochondria | (7) | 18 ± 5 |
| | homogenate | (16) | 19 ± 4 |
| | slices | (25) | 66 ± 8 |
| | myocytes | (10) | 68 ± 7 |

Values are given in % of the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products and are means ± S.D. of the number of experiments given within parentheses.

and rat heart), similar to the results of others (Idell-Wenger *et al.*, 1978; Feuvray & Plouët, 1981). The preparations should predominantly contain mitochondria from subsarcolemmal origin, since no proteolytic enzyme was used.

The oxygen uptake by rat heart mitochondria at 40, 90 and 120 μM palmitate amounted to 446 ± 35, 413 ± 41 and 416 ± 35 natom oxygen/min per mg protein, respectively (means ± S.D. of 3 preparations). Based on a theoretical consumption of 46 natoms oxygen per nmol palmitate these values correspond to oxidation rates of 9-10 nmol palmitate/min per mg protein. The oxygen uptake values are comparable with or higher than those observed by others (Christophersen & Christiansen, 1975; Hansford, 1978; Man & Brosnan, 1982; Olowe & Schulz, 1982) and almost twice as high as for human heart mitochondria (Van Hinsbergh *et al.*, 1979).

With the radiochemical assay, $^{14}CO_2$ production represented only about 4 and 18% of the total palmitate oxidation rate of human and rat heart cell-free preparations, respectively (Table 7.1). In all preparations fatty acid oxidation proceeded at a constant rate during incubation and was proportional to the amount of tissue material. Substrates, coenzymes and cofactors were present at concentrations assuring maximal oxidation rates (Van Hinsbergh *et al.*, 1978a, 1979). When palmitate bound to albumin in a 5:1 molar ratio was used as substrate, the oxidation rate with rat heart

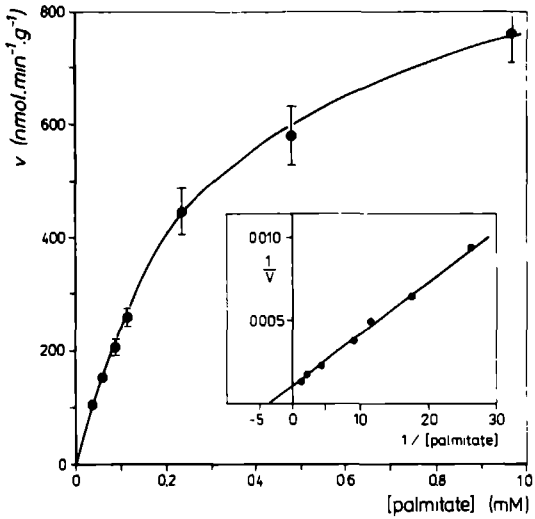



Fig. 7.1. Concentration dependence of palmitate oxidation by rat heart homogenates at 0.4 mM albumin. Values are means ± S.D. of 4 preparations. All concentrations were corrected for substrate utilization according to the method of Lee & Wilson, 1971). Inset figure shows the reciprocal of the palmitate oxidation rate *versus* the reciprocal of the palmitate concentration.

homogenate did not show much dependence on the palmitate concentration in the range of 60-500 µM, but was maximal at 120 µM palmitate. This observation indicates that in homogenates oxidation of fatty acids of endogenous origin hardly influences the radiochemical assay. At a fixed albumin concentration of 0.4 mM, however, palmitate oxidation rose with increasing fatty acid concentration (Fig. 7.1). From the corresponding Lineweaver-Burk plot (inset of Fig. 7.1) the apparent $K_m$ for palmitate was calculated to be 273 ± 35 µM and the maximal oxidation rate 891 ± 100 nmol/min per g (means ± S.D. of 4 determinations). The apparent $K_m$ is comparable to that found with homogenates of diaphragm and of m.quadriceps under these conditions (Fig. 3.6). The maximal oxidation rate appears of the same order of magnitude as that observed with 120 µM palmitate bound to 24 µM albumin (Table 7.2).

The palmitate oxidation rate was in rat heart mitochondria 2.5 -fold and in homogenates 6-fold as high as in the corresponding human heart preparations, when expressed per mg protein and per g muscle, respectively (Table 7.2). The activities of the mitochondrial enzymes cytochrome *c* oxidase and citrate synthase also showed marked differences between human and

*Table 7.2. Palmitate oxidation by cell-free systems of human and rat heart.*

| System | Species | | Oxidation rate (nmol/min) /mg protein or /g muscle | /unit cytochrome *c* oxidase | /unit citrate synthase |
|---|---|---|---|---|---|
| Mitochondria | Man | (5) | 6.68 ± 1.22 | 2.86 ± 0.58 | 6.67 ± 0.72 |
| | Rat | (4-7) | 17.91 ± 1.60 [a] | 2.41 ± 0.35 | 10.25 ± 0.60 [a] |
| Homogenates | Man | (11-15) | 118 ± 23 | 2.22 ± 0.80 | 7.16 ± 1.46 |
| | Rat | (10-16) | 760 ± 107 [a] | 2.30 ± 0.41 | 9.67 ± 1.74 [a] |

Oxidation rates were measured with 90 μM (human mitochondria) or 120 μM (other systems) [1-$^{14}$C]palmitate (bound to albumin in a 5:1 molar ratio). Rates are expressed in nmol/min per mg protein (mitochondria) or per g muscle (homogenates), or relative to the cytochrome *c* oxidase or citrate synthase activity. Values represent means ± S.D. of the number of experiments given within parentheses.
[a] $P < 0.001$, human *versus* rat heart.

*Table 7.3. Activities of mitochondrial enzymes of human and rat heart.*

| System | Species | | Cytochrome *c* oxidase | Citrate synthase | Cytochrome *c* oxidase / citrate synthase ratio |
|---|---|---|---|---|---|
| Mitochondria | Man | (5) | 2.35 ± 0.28 | 1.02 ± 0.23 | 2.38 ± 0.42 |
| | Rat | (7-15) | 7.85 ± 1.04 | 1.59 ± 0.24 | 5.22 ± 1.61 |
| Homogenates | Man | (20-29) | 47.9 ± 12.2 | 17.0 ± 4.1 | 3.00 ± 0.95 |
| | Rat | (28-58) | 327 ± 44 | 77.9 ± 8.3 | 4.12 ± 0.64 |

Enzyme activities are given in units (μmol/min) per mg protein (mitochondria) or per g muscle (homogenates) and represent means ± S.D. of the number of preparations given within parentheses. All values obtained with the rat heart preparations are significantly different from those of human heart ($P < 0.001$).

rat heart (Table 7.3). The differences were larger for cytochrome *c* oxidase than for citrate synthase.

Comparison of the ratio of activities of the inner membrane enzyme cytochrome *c* oxidase and the matrix enzyme citrate synthase in homogenates and mitochondria gives an indication for the intactness of the mitochondria. This ratio did not appear significantly different between mitochondria and homogenates in both species (Table 7.3). Normalization of fatty acid oxidation to mitochondrial marker enzymes is useful since it permits the comparison of this metabolic activity in various cell-free and cellular systems. This normalized activity is also not dependent upon the purity of the mitochondrial preparation. For both species the oxidation rates expressed on the basis of cytochrome *c* oxidase or citrate synthase did not significantly differ between homogenates and mitochondria(Table 7.2). These data confirm that the mitochondria retain their metabolic activity during isolation.

The mitochondrial protein content of human and rat heart can be calculated by comparing the mitochondrial parameters determined in whole homogenates and isolated mitochondria. For rat heart we always used part of the whole homogenate for the isolation of mitochondria, in order to allow a direct comparison. For human heart this was not possible, due to the small size of the biopsies. Mitochondrial content was calculated from the palmitate oxidation rates and cytochrome *c* oxidase and citrate synthase activities (Table 7.4). It appears that the values obtained by means of each of the three parameters are in good agreement with each other and that rat heart has a 2.5-fold higher mitochondrial protein content than human heart. The mitochondrial content of human heart has to our knowledge not yet been reported. For rat heart our values of mitochondrial content are consistent with those reported by others (Abu-Erreisch & Sanadi, 1978; Idell-Wenger *et al.*, 1978; Feuvray & Plouët, 1981)), who used the same enzymes as mitochondrial markers. However, they differ from the higher values calculated on the basis of cytochrome content (Kinnula & Hassinen, 1977).

*Characteristics of the cellular systems*

The intactness of human and rat heart slices was estimated from the leakage of soluble (cytosolic) creatine kinase during the incubation (Wagenmakers & Veerkamp, 1982). After 90 min about 70% of the total soluble creatine kinase activity was still present in the heart slices, indicating

*Table 7.4. Mitochondrial content of human and rat heart.*

| Parameter | Man | Rat |
|---|---|---|
| Palmitate oxidation | 17.7 | 40.5 ± 4.5 |
| Cytochrome *c* oxidase activity | 20.3 | 43.7 ± 8.5 |
| Citrate synthase activity | 16.7 | 49.4 ± 10.7 |

Mitochondrial protein content was calculated as tissue capacity divided by mitochondrial activity of the specific parameters and is given in mg protein/g muscle (wet weight). The values for rat heart represent means ± S.D. of 4 experiments in which part of the whole homogenate was used for the isolation of mitochondria. The mitochondrial content of human heart was calculated using the values given in Tables 7.2 and 7.3.

a small damage of the cells. Estimated by trypan blue exclusion, the intactness of the isolated myocyte preparations was 60-80%. The yield amounted to 2-6 x $10^6$ cells (10-30 mg protein) per g heart tissue. Microscopic examination revealed that 90-95% of the viable cells were elongated, quiescent myocytes, similar in morphology to those found in the intact heart (cf. Fig. 1.2). Cell size ranged from 100 to 150 μm in length and 30 to 40 μm in width. Upon addition of 0.5 - 1 mM $Ca^{2+}$, the cells virtually all maintained their elongated form. After separation on a Percoll gradient about 90% of the cells excluded trypan blue, but in the subsequent centrifugation step, necessary for the collection of the cells, their viability diminished to about 80%. Suspensions of 60-80% viable myocytes were usually applied for the oxidation experiments. For the purpose of comparison, we also studied disrupted myocytes, isolated from the Percoll gradient or prepared by vortexing intact cells.

We tried to establish the percentage of intact (viable) cardiac myocytes in our preparations from the ratio of creatine kinase to citrate synthase activity. During the isolation procedure the myocytes were transferred several times to fresh media, so that cytosolic enzymes (e.g. creatine kinase) from damaged cells are likely to be washed away. Assuming that most of the mitochondria will still remain associated with the myocyte after its disruption (Dow *et al.*, 1981b; Murphy *et al.*, 1982), we determined the ratio of these enzymes in preparations that contained predominantly either viable or non-viable myocytes (based on trypan blue exclusion and separated by density centrifugation). In total heart homogenate the enzyme ratio amoun-

ted to 21.6 ± 0.8, in isolated non-viable myocytes (Percoll gradient) to 13.7 ± 5.6 (means ± S.D. of 6 and 3 preparations, respectively) and in myocyte preparations containing 70 and 75% viable cells to 18.3 and 19.3, respectively. The mean values for non-viable myocytes and total heart homogenate are compatible with the observation that 48% of the cellular creatine kinase resides in the cytosol (Scholte, 1973a). The large variability in the enzyme ratio makes it unsuitable as a viability parameter. This variability may be associated with mechanical damage of our non-viable preparations, since Murphy *et al.* (1982) recently found that cells incubated under anoxic and substrate-deprived conditions released soluble cytoplasmic enzymes in close correlation to trypan blue uptake.

The oxidation rates of $^{14}$C-labeled succinate and L-malate by viable and disrupted myocytes did not differ significantly (data not shown). Such differences were neither observed by Jolly *et al.* (1979) in comparing myocytes and homogenates. These results are not necessarily indicative for cell damage, because the oxidation rates in damaged cells are most likely underestimated due to disruption of citric acid cycle activity, whereas in intact cells the slowly penetrating substrates are completely oxidized (Piper *et al.*, 1982).

*Palmitate oxidation by cellular systems*

Both human and rat heart slices and rat cardiac myocytes oxidized palmitate at a constant rate for at least 90 min (Fig. 7.2). The oxidation rates were not dependent on the amount of tissue material up to 100 mg muscle (slices) or 2 mg protein (myocytes). The contribution of the $^{14}CO_2$ production to the total rate of palmitate oxidation amounted to 33, 66 and 68% for human and rat heart slices and rat cardiac myocytes, respectively (Table 7.1).

Rat heart slices showed higher palmitate oxidation rates than human heart slices, when calculated per g muscle (Table 7.5; page 110). The reverse was found when the rates were expressed relative to the citrate synthase activity, due to the much higher activity of this enzyme in rat heart. The oxidation rates were increased with both human and rat heart slices when higher palmitate:albumin (5:1) concentrations were used (Table 7.5), as earlier observed for rat hemidiaphragm (chapter 3). At a constant albumin concentration of 0.3 mM the oxidation rate by rat heart slices also rose with increasing fatty acid concentration. The apparent $K_m$ for palmi-

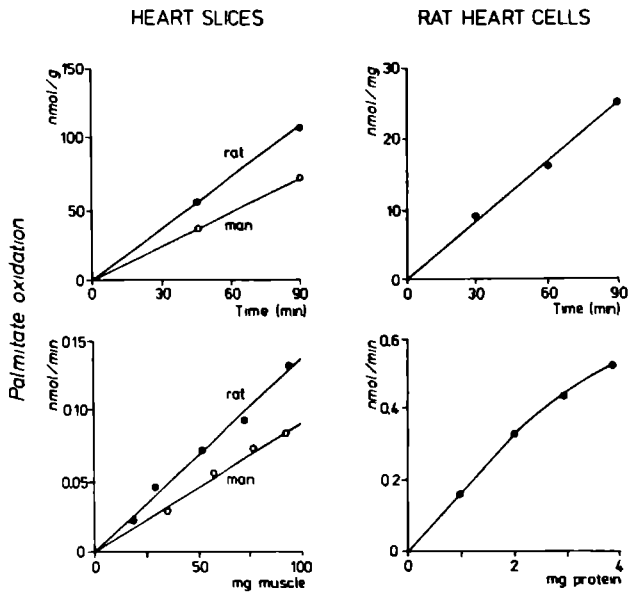



Fig. 7.2. Effect of incubation time and amount of tissue material on the oxidation of 120 μM [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1) by human and rat heart slices and rat heart cells. Results of representative experiments out of three are shown.

tate was calculated to be 1.45 ± 0.41 mM and the maximal oxidation rate 17.3 ± 4.6 nmol/min per g (means ± S.D. of 3 determinations).

The rat cardiac myocyte preparations oxidized palmitate at a rate which was not dependent on the fatty acid concentration and which, when based on the citrate synthase activity, was much higher than with rat heart slices (Table 7.5). With disrupted myocytes, the palmitate oxidation was rather variable (15-35 pmol/min per mg protein) but did not differ for preparations obtained by vortexing or from the Percoll gradient. Because of loss of cytosolic proteins, comparison of the oxidation rates of preparations of intact and disrupted myocytes can better be performed on the basis of citrate synthase activity. Even rounded myocytes retain this enzyme activity (Dow *et al.*, 1981b; Murphy *et al.*, 1982). With disrupted myocytes the palmitate oxidation rate on this basis was about 10% of that for preparations of 60-80% intact myocytes (Table 7.5). This result is similar to findings on myocytes from dog heart (Long *et al.*, 1980). For our cell preparations, containing 60-80% viable cells, the contribution of the non-viable

*Table 7.5. Palmitate oxidation by cellular systems of human and rat heart.*

| Species | System | Concentration (mM) | | Oxidation rate (nmol/min) /g muscle or /mg protein | Oxidation rate (nmol/min) /unit citrate synthase |
|---|---|---|---|---|---|
| Man | Slices | 0.12 | (7) | 0.91 ± 0.08 | 0.054 ± 0.005 |
| Rat | Slices | 0.12 | (22) | 1.44 ± 0.23 [c] | 0.019 ± 0.003 [c] |
| | | 1.5 | (3) | 8.12 ± 0.53 | 0.108 ± 0.007 |
| | Myocytes [a] | 0.12 | (7) | 0.31 ± 0.07 | 0.41 ± 0.08 |
| | | 0.6 | (3) | 0.27 ± 0.03 | 0.35 ± 0.06 |
| | Disrupted myocytes [b] | 0.12 | (5) | 0.025 ± 0.009 | 0.041 ± 0.004 |

Oxidation rates were measured with [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1) and are expressed in nmol/min per g muscle (slices) or per mg protein (myocytes), or relative to the citrate synthase activity. Values represent means ± S.D. of the number of experiments given within parentheses.

[a] Preparations that contain 60-80% viable cells (based on trypan blue exclusion.

[b] Disrupted by vortexing or obtained from the Percoll gradient.

[c] $P < 0.001$, human *versus* rat heart slices.

cells to the total palmitate oxidation rate will, therefore, only amount to 2-4%. Because of the lack of a good viability standard, no attempt was made to recalculate the oxidation rates of myocytes by extrapolation to 100% viability, as suggested by Long *et al.* (1980), so that our values were slightly underestimated.

Various factors, involved in fatty acid oxidation, were tested for their effect on palmitate oxidation by the cellular preparations (Table 7.6). Carnitine, with or without coenzyme A, stimulated the oxidation in slices, myocytes and disrupted myocytes by about 40%, similar to our observations with intact skeletal muscle preparations (chapter 3). Addition of ATP gave a comparable stimulation. When these three factors were simultaneously present, the palmitate oxidation rate was dramatically increased with

*Table 7.6. Effect of various additions on palmitate oxidation by heart slices and cardiac myocytes.*

| Addition | Human heart slices | Rat heart | | |
|---|---|---|---|---|
| | | Slices | Myocytes [a] | Disrupted myocytes [b] |
| None | 100 | 100 | 100 | 100 |
| 4 mM D,L-carnitine and 0.1 mM CoASH | - | 143 ± 16 | 139 ± 18 | 146 ± 39 |
| 5 mM ATP | - | 140 ± 26 | 125 ± 5 | 167 ± 22 |
| 1 mM L-malate | - | - | 63 ± 15 | 61 ± 27 |
| 4 mM D,L-carnitine, 0.1 mM CoASH and 5 mM ATP | 200 ± 40 | 435 ± 31 | 809 ± 103 | 3650 ± 324 |
| 4 mM D,L-carnitine, 0.1 mM CoASH, 5 mM ATP and 1 mM L-malate | - | - | 897 ± 108 | 5181 ± 1030 |

Relative oxidation rates are given in % of the corresponding control and are means ± S.D. of 3-7 paired experiments. The control values were 0.96 ± 0.06 and 1.44 ± 0.24 nmol/min per g muscle for human and rat heart slices, respectively, and 0.31 ± 0.07 and 0.028 ± 0.006 nmol/min per mg protein for myocytes and disrupted myocytes, respectively (means ± S.D. of 3, 20, 7 and 4 preparations).

[a] Preparations that contain 60-80% viable cells (based on trypan blue exclusion

[b] Disrupted by vortexing or obtained by gradient centrifugation on Percoll.

all preparations, but especially with disrupted myocytes. With myocytes and disrupted myocytes this increase was even larger when L-malate was also added to the medium. This synergistic effect most likely relates to palmitate activation and supply of citric acid cycle metabolites, since especially the production of $^{14}C$-labeled acid-soluble intermediates increased (data not shown). The 8-fold increase of palmitate oxidation observed with myocyte preparations, containing 60-80% trypan blue-excluding cells, can only partly be attributed to a higher oxidation rate in the damaged cells. If carnitine, coenzyme A and ATP are added to the medium, the contribution of the non-viable cells to the total palmitate oxidation rate of these preparations can be calculated to rise to 8-16%. Apparently, the viable myocytes also oxidize palmitate at a higher rate (6-7-fold) when the three cofactors are added. It is known that both carnitine and ATP, which may have been re-

leased during the isolation of the cells, can be taken up by intact cardiac myocytes (Bahl *et al.*, 1980; Chaudry, 1982). Liu & Spitzer (1978) and Long *et al.* (1980) also reported a stimulatory effect of carnitine on the $^{14}CO_2$ production from [1-$^{14}$C]palmitate by viable dog heart myocytes. The observed effect of ATP is not exerted by supplying adenosine nor inosine as these compounds did not stimulate the oxidation rates in rat heart slices, not even in the presence of carnitine and coenzyme A.

*Lipolysis studies*

In order to estimate to what extent fatty acids of endogenous origin may influence the oxidation of exogenous substrates, we measured the rate of endogenous fatty acid production in rat heart slices. This rate can be approximated by assay of the glycerol release into the medium, since endogenous fatty acids are mainly derived from intermyofibrillar triacylglycerol pools and glycerol kinase activity is almost absent in the heart (Newsholme & Taylor, 1969). Glycerol was released at a constant rate for at least 40 min. At 0°C the release was less than 20% of that measured at 37°C. In the absence of added substrate the glycerol release amounted to 20.5 ± 2.5 nmol/min per g (mean ± S.D. of 3 preparations). This value is twice as high as found in perfused rat hearts (Hulsmann *et al.*, 1981). The release was not affected by the addition of 11 mM glucose to the incubation medium, but was lower with 120 μM palmitate. The relative values were 101 ± 4 and 85 ± 6%, respectively (means ± S.D. of 3 paired experiments). When the palmitate oxidation rate was measured in heart slices, which had first been used for the release experiments, a comparable oxidation rate was observed as without such preincubation. The inhibitory effect of palmitate appears rather small when compared to studies with perfused heart (Crass *et al.*, 1975) or purified lipase preparations (Severson & Hurley, 1982). This may be due to a hampered diffusion of the fatty acid within the heart slices and to the absence of hormones. Endogenous fatty acids are produced at a sufficiently high rate to cause label dilution of the added |$^{14}$C|palmitate and hence underestimation of the fatty acid oxidation rate. The linearity with time indicates a nearly immediate equilibration of the pools of external and endogenously formed fatty acids.

## 7.4. DISCUSSION

The four *in vitro* systems investigated in this study, homogenates, isolated mitochondria, slices and cardiac myocytes, differ considerably with respect to ease of preparation, complexity and, as was shown, metabolic properties. They all appeared to be valid *in vitro* systems. The cell-free systems were established to contain functionally intact mitochondria. In the cellular systems a small part of the cells is not intact, but from the observed linearity of the fatty acid oxidation with time it appears that the cells retain their viability for sufficient periods of time to permit valid metabolic studies.

With isolated rat heart mitochondria fatty acid oxidation was measured polarographically as well as radiochemically with non-limiting amounts of fatty acids. Under these conditions palmitate molecules are not completely degraded to $CO_2$ and hence accumulation occurs of oxidation intermediates, mainly citric acid cycle metabolites and short-chain acylcarnitines (Christophersen & Christiansen, 1975; Van Hinsbergh *et al.*, 1978a, 1979; cf. Fig. 5.1 on page 81). When only $^{14}CO_2$ production (3.2 nmol/min per mg protein) is considered the palmitate oxidation rate is about 30% of that calculated from oxygen uptake (9-10 nmol/min per mg protein). The palmitate oxidation rate estimated from the sum of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products (18 nmol/min per mog protein) is about 80% higher than when calculated from oxygen uptake. This discrepancy may be explained by the fact that in the radiochemical assay all oxidation intermediates are equally considered, whereas in the polarographical assay the intermediates are quantified dependent on the amount of reducing equivalents released during their formation. We conclude that in measuring the β-oxidation of [$^{14}C$]fatty acids the sum of $^{14}CO_2$ and $^{14}C$-labeled acid-soluble products is a more accurate and sensitive index than the $^{14}CO_2$ production alone. Furthermore, when calculating palmitate oxidation rates from the rate of oxygen uptake, the conversion factor to be used is generally lower than 46. Comparison of the oxygen uptake rate (430 natom/min per mg protein) and the radiochemically determined oxidation rate (18 nmol/min per mg protein) shows that with rat heart mitochondria about 24 natom oxygen are consumed per nmol palmitate oxidized under our conditions.

The palmitate oxidation rates measured in cell-free systems represent the best approximation of values of the fatty acid oxidation capacity. The oxidation capacities of human heart have previously not been determined. The palmitate oxidation capacity of rat heart homogenates is somewhat higher than the values based on oxygen consumption in homogenates (Hooker & Baldwin, 1979) and perfused non-working heart (Hülsmann *et al.*, 1982), but it agrees quite well with the rate of β-oxidation (Oram *et al.*, 1973; Pearce *et al.*, 1979) and of oxygen consumption (Taegtmeyer *et al.*, 1980) in the perfused working rat heart. A lower oxidation rate was reported for oleate in rat heart homogenate (McGarry *et al.*, 1978a). Earlier we determined palmitate oxidation rates in mitochondrial preparations of human heart muscle which were obtained from patients with tetralogy of Fallot (Van Hinsbergh *et al.*, 1979). Since our present values, measured in heart auricular biopsies, are not different from those previously reported, we confirm that tetralogy of Fallot does not seem to be accompanied by dysfunction of cardiac mitochondria (Lentz *et al.*, 1978).

Our values for the cytochrome *c* oxidase and citrate synthase activities in rat heart are comparable to those found by others in mitochondria (Palmer *et al.*, 1977; Idell-Wenger *et al.*, 1978; Feuvray & Plouët, 1981; Matlib *et al.*, 1981) and homogenates (Baldwin *et al.*, 1977; Idell-Wenger *et al.*, 1978; Feuvray & Plouët, 1981; Hansford & Castro, 1982).

At comparison of our values for human and rat heart one has to keep in mind possible effects of sample bias, i.e. human auricular biopsy *versus* entire rat heart. Ventricular myocardial cells of the rat were reported to contain a larger mitochondrial volume than atrial cells (Hirakow *et al.*, 1980), although earlier no differences were found (Nakata, 1977). The free fatty acid concentration in dog right auricular appendages was higher than in their left ventricular wall, suggesting a lower metabolic activity in the former (Van der Vusse *et al.*, 1983). The percentage of non-muscle cells may also differ in the human and rat heart preparations. The concentrations of collagen and RNA in the atria of heart are considerably greater than those in the ventricles (Caspari *et al.*, 1975; Smith & Sugden, 1983).

The 6-fold higher palmitate oxidation rate in homogenates of total rat heart muscle in comparison to human atrial muscle is not only due to a 2.5-fold higher content of mitochondrial protein but also to a 2.5-fold higher mitochondrial activity. The relatively low contribution of $^{14}CO_2$

production to the total rate of palmitate oxidation in cell-free systems in human heart compared to rat heart (Table 7.1) indicates that the limitation of the citric acid cycle with respect to the rate of β-oxidation is more prominent in human heart.

We are not familiar with fatty acid oxidation rates in heart slices. Reported values for oxidation rates by cardiac myocytes were always only based on $^{14}CO_2$ production. Our values for $^{14}CO_2$ production from [1-$^{14}$C]palmitate by rat cardiac myocytes (0.18 - 0.22 nmol/min per mg protein) agree quite well with the results of most other investigators (e.g. Grosso *et al.*, 1977; De Grella & Light, 1980b; Frangakis *et al.*, 1980), but are lower than found by Montini *et al.*(1981). Myocytes from dog heart show a $^{14}CO_2$ production of 0.10 - 0.14 nmol/min per mg protein (Liu & Spitzer, 1978; Vahouny *et al.*, 1979; Long *et al.*, 1980). Recently, Tamboli *et al.* (1983) reported a $^{14}CO_2$ production from [1-$^{14}$C]oleate by rat cardiac myocytes of 0.08 nmol/min per mg protein. From their data we calculated the production of $^{14}$C-labeled water-soluble products and thus found the total oleate oxidation rate to be 0.14 nmol/min per mg protein, the $^{14}CO_2$ production of which is 58%. The latter percentage agrees with our findings for palmitate.

Fatty acid oxidation rates of cell-free and cellular systems can be compared, when they are expressed relative to the citrate synthase activity. On this basis the oxidation rates of the cellular systems are only a few percent of those of the cell-free preparations (Tables 7.2 and 7.5). This difference is related to an underestimation of the oxidation rates of the cellular systems due to label dilution by endogenous fatty acid and to the low metabolic activity of these resting cellular preparations (Taegtmeyer *et al.*, 1980). The lowest oxidation rates were measured in heart slices. This can be explained by slow diffusion of the fatty acid in the tissue, since with slices the oxidation rate increases with the fatty acid concentration, in contrast to cardiac myocytes of dog (Liu & Spitzer, 1978) and rat (Table 7.5).

In addition, we found less difference between the oxidation rates in slices and myocytes for pyruvate than for palmitate (data not shown). The diffusion and/or the presence of stored lipids appeared to affect more the palmitate oxidation rates in rat than in human heart slices, since the rate bsed on citrate synthase activity is lower in rat heart slices (Table 7.5) but significantly higher in homogenates (Table 7.2) than in the correspon-

ding human preparations.

Cell-free systems are useful for the study of metabolic capacities of tissues and of regulatory phenomena at the subcellular level. Mainly because of the large dilution of the cytoplasmic pool in these preparations, endogenous substrates hardly influence the oxidation of exogenously added substrates. This dilution effect probably also explains why fatty acids are less completely degraded in cell-free than in cellular systems, as follows from the observation that the contribution of the $^{14}CO_2$ production to the total palmitate oxidation rate was much smaller in cell-free systems (Table 7.1).

Effects of endogenously derived substrates should be considered, when studying cellular systems. Investigations on the fatty acid oxidation in slices appeared to be hampered by intercellular transport of the fatty acid and possibly also by contributions of non-muscle cells. Isolated myocytes appear to be the most useful for the investigation of metabolic regulatory mechanisms at the cellular level. For the elucidation of the regulation of fatty acid metabolism in human heart muscle, further comparative studies, using isolated myocytes and homogenates, will be fruitful. At the moment the amounts of tissue available still impair the isolation of sufficient quantities of intact human heart cells.

## 7.5. SUMMARY

Palmitate oxidation rates and activities of the mitochondrial marker enzymes cytochrome *c* oxidase and citrate synthase have been determined in homogenates, isolated mitochondria and slices of human and rat heart and in calcium-tolerant rat cardiac myocytes. Homogenates and mitochondria from rat heart showed a 6- and 2.5-fold higher palmitate oxidation rate than the corresponding preparations from human heart.

From the palmitate oxidation rates and cytochrome *c* oxidase and citrate synthase activities as parameters, the mitochondrial protein contents of human and rat heart were calculated to be about 18 and 45 mg/g wet weight, respectively.

Based on citrate synthase activities, the palmitate oxidation rates were about the same in homogenates and isolated mitochondria, much lower in myocytes and lowest in slices. Palmitate oxidation rates were concentration

dependent in slices but not with myocytes.

With the cellular systems palmitate oxidation was synergistically stimulated by the addition of carnitine, coenzyme A and ATP to the medium. This stimulation could only partly be attributed to an increased oxidation in damaged cells.

# CHAPTER 8

# A RADIOCHEMICAL PROCEDURE FOR THE ASSAY OF FATTY ACID BINDING BY PROTEINS*

## 8.1. INTRODUCTION

The cytosol of many eucaryotic cells contains one or more fatty acid-binding proteins, which exhibit a high affinity for long-chain fatty acids and are assumed to participate in the intracellular transport of fatty acids (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). These proteins are usually detected by coelution of labeled fatty acids from a gel-filtration column. This technique, however, is not very sensitive, since fatty acids also bind with a high affinity to the gel itself (cf. Mishkin *et al.*, 1975). Therefore, a more useful and sensitive method for the detection of fatty acid-binding proteins is greatly needed.

Dahlberg *et al.* (1980) recently described the use of Lipidex 1000 (a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25) (Dyfverman & Sjövall, 1978) to remove unbound as well as protein-bound hydrophobic molecules from aqueous solutions in a temperature-dependent manner according to the kinetics of protein-lipid interaction. Lipidex 1000 treatment at 2-4$^{o}$C resulted in the separation of unbound steroids from protein-steroid complexes, whereas at 45$^{o}$C all steroids were removed (Dahlberg *et al.*, 1980). In the present work we show that Lipidex 1000 can also be used for the temperature-dependent removal of fatty acids from protein-fatty acid complexes and for the detection and assay of fatty acid-binding proteins. The procedure was applied to assay the fatty acid binding of some proteins and the maximal binding capacity of bovine serum albumin and of cytosolic proteins from rat liver.

*Adapted and extended from Glatz & Veerkamp (1983a).

*Methods*

Lipidex 1000 was washed free of methanol in a column by elution with 10 bed volumes of 10 mM K-phosphate (pH 7.4), containing 0.01% $NaN_3$. The gel was transferred to a glass vial and stored at 4°C as a Lipidex-buffer suspension (1:1, v/v) containing about 100 mg dry Lipidex/ml buffer.

*Delipidation by Lipidex chromatography*

The removal of protein-bound fatty acids by Lipidex was examined with complexes of [1-$^{14}$C]palmitate and bovine serum albumin and of palmitate and [$^{14}$C]methylated bovine serum albumin, both in a 5:1 molar ratio. The protein sample was applied to a column of Lipidex (0.7 x 6 cm), equilibrated with 10 mM K-phosphate buffer (pH 7.4) at 37°C (thermostated column; flow rate, 12 ml/h). Elution was performed with 4 bed volumes of the same buffer, whereafter methanol was used to elute adsorbed compounds.

*Assay of fatty acid binding*

An aqueous solution of about 5 µM [1-$^{14}$C]palmitate (pH 8) was prepared fresh. In a round-bottom flask, 50 nmol [1-$^{14}$C]palmitate (2.8 µCi) was dissolved in 8 ml ethanol and mixed with 8 ml water. The pH of the solution was adjusted to 8 with 0.2 M KOH. Ethanol was removed by rotary evaporation under low pressure at 25°C. About 4 ml of water was added to compensate for its loss by evaporation. The ethanol content of the resulting solution was determined with a Boehringer Test Combination (Boehringer, Mannheim, F.R.G.) and kept below 0.5%.

For the standard assay of fatty acid binding, protein samples were incubated in 1.5-ml polyethylene vials in 10 mM K-phosphate (pH 7.4) with 1 µM [1-$^{14}$C]palmitate. The total volume was 0.45 ml. After incubation for 10 min at 37°C the vials were cooled in ice and 0.05 ml of ice-cold stirring Lipidex-buffer suspension was added to remove the unbound fatty acids. The samples were mixed thoroughly, incubated for 10 min at 0°C and centrifuged in a Burkard Koolspin microcentrifuge (2 min, 10,000 x *g*) at 4°C. An aliquot of the supernatant was assayed for radioactivity by liquid scintillation counting in Opti-Fluor. Fatty acid binding was calculated from the amount of radioactivity present in the supernatant and is expressed as pmol/µg protein. For comparison, samples were in some experiments treated

with 0.05 ml of a buffer suspension of dextran-coated charcoal (2% (w/v) charcoal, 0.2% (w/v) Dextran T-70).

*Preparation of rat liver cytosol*

Rat liver cytosol was prepared as described in section 2.3 and freed of residual albumin by passage through an affinity column of the immunoglobulin G fraction of rabbit anti-rat albumin antiserum coupled to Protein A-Sepharose CL-4B. For delipidation, 4 ml of the cytosol, containing about 30 mg protein, was subjected to chromatography on Lipidex 1000 at 37°C essentially as described above, except that the column used was 1 x 15 cm and the flow rate 20 ml/h. The resulting delipidated protein samples were stored at -20°C. Protein content was determined as described in section 2.3.

## 8.3. RESULTS

*Temperature-dependent interaction of palmitate-albumin complexes and Lipidex 1000*

When an aqueous solution of bovine serum albumin and [1-$^{14}$C]palmitate was incubated with Lipidex at 0°C, a fixed amount of palmitate (about 35% of the added fatty acid) could not be precipitated by the gel, even after a 60-min incubation (Fig. 8.1). This portion presumably represents albumin-bound palmitate. However, when the incubation was performed at 37 or 45°C, 88-94% of this amount of palmitate was bound by Lipidex after 60 min (Fig. 8.1), indicating that at these temperatures albumin-bound fatty acids were also removed from the solution. These observations extend those of Dahlberg *et al.* (1980) in that Lipidex 1000 is also suitable for the removal of unbound and/or protein-bound fatty acids from aqueous solutions in a temperature-dependent way.

The removal of protein-bound fatty acids was also studied using a column of Lipidex 1000 at 37°C. [1-$^{14}$C]Palmitate (30 nmol) complexed to bovine serum albumin (molar ratio 5:1) was applied to the column and elution performed with buffer and subsequently methanol. The buffer fraction contained only 2.2 ± 0.5% of the applied radioactivity while the methanol fraction contained 95.8 ± 0.8% (means ± S.D. of 3 experiments).

Recovery of protein from the column was examined by chromatography at 37°C of a complex of 2 nmol palmitate to 0.4 nmol *N*-[$^{14}$C]methylated bovine serum albumin. The buffer and methanol fractions contained 98 ± 10 and

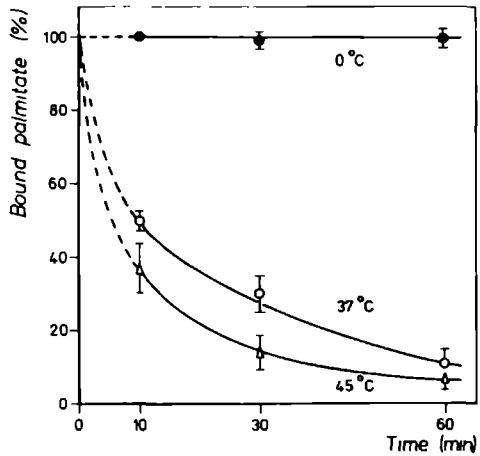



Fig. 8.1. Temperature dependence of the removal of fatty acids from albumin and binding to Lipidex 1000. The binding of 1 μM [1-$^{14}$C]palmitate to 5 μg bovine serum albumin was measured with the standard assay except that after addition of Lipidex the samples were incubated at 0°C (●), 37°C (o) or 45°C (Δ). The amount of palmitate bound to albumin after 10 min at 0°C (32 pmol/μg albumin) was set at 100%. Values are means ± S.D. of 3 experiments.

0.4 ± 0.2% of the applied radioactivity, respectively (means ± S.D. of 4 determinations). The recovery of native bovine serum albumin after Lipidex chromatography at 37°C amounted to 94.3 ± 3.1% (mean ± S.D. of 4 determinations). Also, with a rat liver 105,000 × *g* supernatant, protein recovery after chromatography was 91 and 96% (2 determinations). These results show that delipidation of protein-fatty acid complexes can be performed effectively by Lipidex 1000 chromatography at 37°C, without loss of protein. A temperature of 37°C is preferred over 45°C (Dahlberg *et al.*, 1980) to minimize possible conformation changes or denaturation of protein.

*Assay of fatty acid binding: comparison of the use of Lipidex 1000 and dextran-coated charcoal*

For the assay of fatty acid binding, protein samples were incubated with 1 μM [1-$^{14}$C]palmitate at 37°C. This relatively low concentration of palmitate was chosen because of its low solubility in aqueous solutions. After equilibration the unbound fatty acids were removed from the aqueous solution using Lipidex at 0°C. Under our standard assay conditions, a linear correlation between the amount of fatty acid bound and the amount of

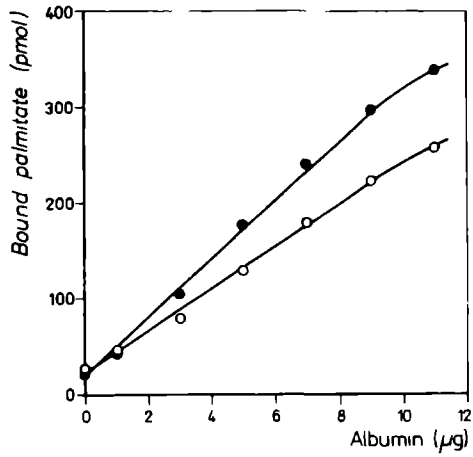



Fig. 8.2. Effect of the amount of bovine serum albumin on palmitate binding. Determinations were carried out with the standard assay. Various amounts of albumin were incubated with 1 μM [1-$^{14}$C]palmitate for 10 min at 37°C, followed by an incubation with Lipidex 1000 (●) or dextran-coated charcoal (o) for 10 min at 0°C. Results of one representative experiment out of three are shown.

protein was observed up to about 10 μg albumin, or 50-60% of the added palmitate (Fig. 8.2). Fatty acid binding was not influenced by the amount of Lipidex used (Fig. 8.3). The blank value amounted only to 15-25 pmol (Fig. 8.2), indicating that 95-97% of the unbound fatty acids can be removed from aqueous solutions. The polyethylene vial could interfere with the assay by also binding palmitate, but this binding is negligible at short incubation times and at pH values above 7 (Sallee, 1974). Under our assay conditions (10-min incubation and pH 7.4), fatty acid binding to the polyethylene appeared to be low, since the recovery of [1-$^{14}$C]palmitate from an assay mixture without protein amounted to 91 ± 5% (mean ± S.D. of 11 determinations). In the presence of 5 and 10 μg serum albumin the recovery was 93 ± 2 and 95 ± 3%, respectively (means ± S.D. of 4 determinations), indicating that the polyethylene-bound fatty acids are still available for binding by protein. The palmitate-binding capacity of Lipidex at 0°C was 0.7 - 0.8 nmol/50 μl Lipidex-buffer suspension (28-32 nmol/ml packed gel).

For comparison, properties of dextran-coated charcoal were also investigated. This material is commonly used for the separation of protein-bound and unbound hydrophobic molecules (Warner & Neims, 1975; Benassayag

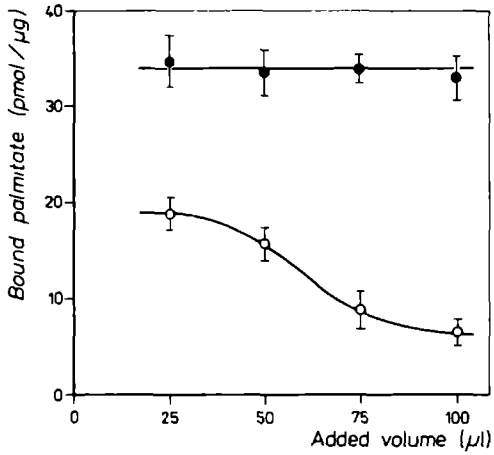



Fig. 8.3. Effect of the amount of Lipidex 1000 (●) or dextran-coated charcoal (o) suspension on the binding of palmitate by bovine serum albumin, as measured with the standard assay. After incubation of 5 µg protein with 1 µM [1-$^{14}$C]palmitate for 10 min at 37°C, various amounts of Lipidex 1000 or dextran-coated charcoal were added and the solutions were incubated for 10 min at 0°C. Values are means ± S.D. of 3 experiments.

*et al.*, 1977; Dahlberg *et al.*, 1980). The dextran-coated charcoal suspension used was chosen so that its palmitate-binding capacity per ml was comparable to that of the Lipidex-buffer suspension. With dextran-coated charcoal we observed a similar linear dependence on the amount of protein as with Lipidex (Fig. 8.2). The amount of bound palmitate recovered in the supernatant was, however, about 30% lower and decreased considerably when larger quantities of dextran-coated charcoal were used (Fig. 8.3).

To examine the possible binding of protein to Lipidex and dextran-coated charcoal at 0°C, assays were performed with *N*-[$^{14}$C]methylated bovine serum albumin and *N*-[$^{14}$C]methylated ovalbumin, in the presence or absence of unlabeled palmitate. The hydrophobic properties of bovine serum albumin were hardly affected by methylation of the free amino groups (O'Hare & Nice, 1979). Table 8.1 shows that when no palmitate was present, the recovery of protein was 90-99% for Lipidex (both with albumin and ovalbumin), but only 66-87% for dextran-coated charcoal (albumin). In the presence of 1 µM palmitate the recovery of 5 µg [$^{14}$C]methylated bovine serum albumin was 4.76 ± 0.17 and 3.74 ± 0.22 µg for Lipidex and dextran-coated charcoal,

*Table 8.1. Recovery of protein after treatment with Lipidex 1000 or dextran-coated charcoal.*

| Added protein (μg) | Added Lipidex or charcoal suspension (μl) | Recovered protein (μg) | | |
|---|---|---|---|---|
| | | Bovine serum albumin | | Ovalbumin |
| | | Lipidex 1000 | Dextran-coated charcoal | Lipidex 1000 |
| 1 | 50 | 0.95 ± 0.03 | 0.82 ± 0.04 | 0.93 ± 0.08 |
| 2.5 | 50 | 2.44 ± 0.28 | 1.99 ± 0.35 | 2.24 ± 0.06 |
| 5 | 25 | 4.55 ± 0.07 | 3.69 ± 0.10 | 4.49 ± 0.10 |
| 5 | 50 | 4.77 ± 0.41 | 4.02 ± 0.62 | 4.44 ± 0.16 |
| 5 | 75 | 4.82 ± 0.14 | 3.77 ± 0.66 | 4.74 ± 0.09 |
| 5 | 100 | 4.81 ± 0.07 | 3.31 ± 0.11 | 4.61 ± 0.10 |
| 10 | 50 | 9.86 ± 0.09 | 8.71 ± 0.38 | 9.51 ± 0.31 |

Various quantities of *N*-[$^{14}$C]methylated bovine serum albumin or *N*-[$^{14}$C]methylated ovalbumin were incubated in 10 mM K-phosphate buffer (pH 7.4) for 10 min at 37°C. After cooling in ice an amount of a Lipidex or dextran-coated charcoal suspension in buffer was added to make a final volume of 0.5 ml. The sample was mixed thoroughly, incubated for 10 min at 0°C, and centrifuged (2 min, 10,000 × *g*). The recovery of protein was calculated from the amount of $^{14}$C radioactivity present in the supernatant. Values represent means ± S.D. of 4 experiments.

respectively (means ± S.D. of 3 experiments).

Thus, at 0°C, Lipidex 1000 selectively binds unbound fatty acids with a high effectiveness, whereas with dextran-coated charcoal the selectivity and effectiveness are much less.

*Fatty acid binding of some proteins*

Human, bovine and rat serum albumins show similar binding properties for palmitate (Table 8.2). The binding of [1-$^{14}$C]oleate was also similar for human and bovine serum, but was lower for rat serum albumin (Table 8.2). Human and bovine serum albumin have earlier been found to exhibit a slightly higher affinity for oleate than for palmitate (Spector, 1975). The capacity of myoglobin to bind palmitate is comparatively low. Therefore, the suggestion of Gloster & Harris (1977) that myoglobin serves as a fatty acid carrier in muscle appears improbable (cf. chapter 11). The fatty acid bin-

*Table 8.2. Palmitate and oleate binding by various proteins.*

| Protein | Source | Fatty acid binding (pmol/µg) | |
|---|---|---|---|
| | | Palmitate | Oleate |
| Albumin | Human serum | 32.1 ± 1.9 | 38.1 ± 1.7 |
| | Bovine serum | 32.9 ± 1.6 | 37.7 ± 1.7 |
| | Rat serum | 30.1 ± 2.7 | 27.7 ± 1.7 |
| Myoglobin | Horse heart | 3.5 ± 1.4 | - |
| | Horse skeletal muscle | 3.0 ± 1.0 | - |

Binding measurements were performed with the standard assay, using 1 µM [1-$^{14}$C]palmitate or [1-$^{14}$C]oleate and 5 µg protein per assay. Values represent means ± S.D. of at least 4 experiments.

ding of bovine serum albumin is hardly dependent on the composition of the assay medium at pH 7 - 8.5, but is substantially lower at pH 5.8 (Table 8.3).

*Estimation of the maximal binding capacity of bovine serum albumin and of cytosolic proteins of rat liver*

The described assay procedure was employed to examine the maximal capacity of fatty acid binding by bovine serum albumin. Assays were performed with a fixed amount of protein (0.5 - 5 µg) at various concentrations of

*Table 8.3. Effect of the assay medium on palmitate binding by albumin.*

| Assay medium | Palmitate binding (pmol/µg) |
|---|---|
| 10 mM K-phosphate, pH 7.4 | 32.9 ± 1.6 |
| 50 mM K-phosphate, pH 5.8 | 21.6 ± 1.7 |
| pH 6.5 | 27.8 ± 3.4 |
| pH 7.4 | 33.5 ± 3.0 |
| pH 8.5 | 33.4 ± 4.3 |
| 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4 | 39.3 ± 3.7 |
| 154 mM NaCl | 34.2 ± 3.6 |

The binding of 1 µM [1-$^{14}$C]palmitate to 5 µg bovine serum albumin was measured with the standard assay, using the indicated media. Values are means ± S.D. of at least 4 experiments.

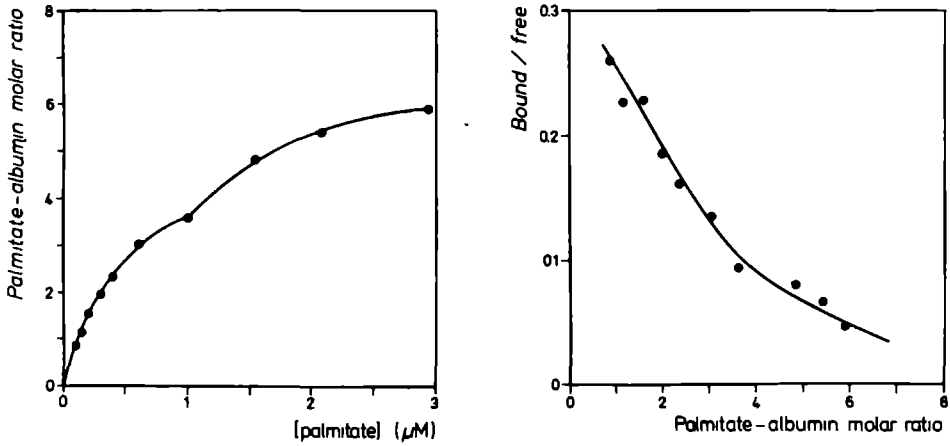



Fig. 8.4. Palmitate binding by bovine serum albumin as a function of the total palmitate concentration. Assays were performed with 1 μg protein and various concentrations of [1-$^{14}$C]palmitate, according to the standard procedure, as described under Materials and Methods. The binding curve (left) was analyzed by the method of Scatchard (1949) (right). The concentration of free fatty acids was calculated from the initial concentration and the amount of protein-bound fatty acid. No correction was applied for fatty acid binding to the polyethylene vial. The results are shown of a representative experiment out of eight.

[1-$^{14}$C]palmitate (0.1 - 3 μM) and binding data were analyzed by the method of Scatchard (1949) (Fig. 8.4). The curvilinear Scatchard plot is comparable to the one obtained by equilibrium-dialysis studies (Spector, 1975). The apparent dissociation constants were calculated to range from 0.3 to 1.2 μM (8 experiments), which correlates with the values reported for the individual fatty acid-binding sites of albumin (Spector, 1975). The results show that with the present assay relevant fatty acid-binding data can be obtained.

The fatty acid-binding capacity of dealbuminized rat liver cytosol was investigated in the same way. Fatty acid binding proceeded linearly with the amount of protein (cf. Fig. 10.1) and did not decrease after storage at -20°C (up to 6 months). Scatchard plot analysis (Fig. 8.5) revealed an apparent dissociation constant $K_d$ of 1.01 ± 0.16 μM and a maximum palmitate binding of 4.39 ± 0.26 pmol/μg protein (means ± S.D. of 5 preparations, using 20-30 μg protein per assay).

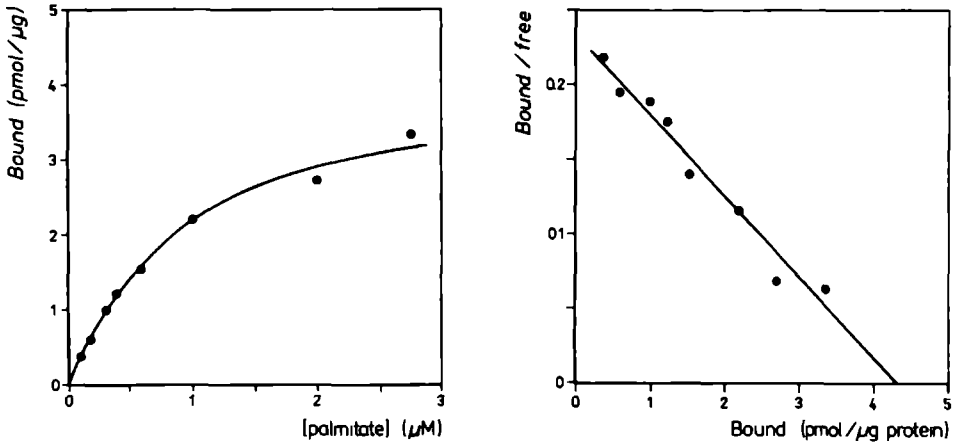



Fig. 8.5. Palmitate binding by the dealbuminized and delipidated 105,000 × *g* supernatant of rat liver as a function of the total palmitate concentration. Assays were performed and analyzed as described in the legend to Fig. 8.4, except that the amount of protein used per assay was 23 µg. The results of a representative experiment are shown. The correlation coefficient *r* and the apparent dissociation constant $K_d$ were 0.93 ± 0.06 and 1.01 ± 0.16 µM, respectively (means ± S.D. of 5 preparations).

Fatty acid binding by cytosolic protein was significantly lower without delipidation when assayed at 0.2 µM, but not at 1 µM [1-$^{14}$C]palmitate (Table 8.4). The competition of endogenous fatty acids is more pronounced at a low palmitate concentration. Owing to this effect we obtained curvilinear Scatchard plots with cytosol preparations that had not been delipidated (cf. Fig. 10.2). A similar effect of delipidation was observed with steroid receptor studies (Dahlberg *et al.*, 1980). After a second delipidation the fatty acid binding did not change further at both palmitate concentrations (data not shown), indicating that the binding capacity of the protein samples was not influenced by delipidation.

## 8.4. DISCUSSION

Lipidex 1000 is a derivative of Sephadex G-25 which contains a sufficiently large number of alkyl chains to give it the properties of an organic solvent or nonpolar adsorbent, while still being sufficiently polar to be wetted by water (Ellingboe *et al.*, 1970; Dyfverman & Sjövall, 1978).

*Table 8.4. Effect of delipidation on palmitate binding by rat liver cytosolic proteins.*

| Palmitate concentration (μM) | Palmitate binding (pmol/μg protein) | |
|---|---|---|
| | Before delipidation | After delipidation |
| 0.2 | 0.68 ± 0.15 | 0.92 ± 0.17 [a] |
| 1.0 | 2.17 ± 0.38 | 2.27 ± 0.27 [b] |

Dealbuminized rat liver cytosol was delipidated by Lipidex 1000 chromatography at 37°C. Binding measurements were performed with the standard assay, using 0.2 or 1 μM [1-$^{14}$C]palmitate and 20-30 μg cytosolic protein. Values are means ± S.D. of 6 preparations. Values obtained before and after delipidation are compared by the unpaired t-test.

[a] $P < 0.05$

[b] Not significant

The gel was found to exhibit a high affinity for unbound long-chain fatty acids at both 0 and 37°C, and also showed an affinity for protein-bound fatty acids, but only at 37 or 45°C. These observations are explained by the kinetics of protein-lipid interaction, according to which protein-bound fatty acids are more available for removal at a higher temperature. We applied this property of Lipidex for the delipidation of protein samples as well as for the assay of fatty acid binding by proteins, as is schematically illustrated in Fig. 8.6.

For delipidation of protein samples, Lipidex 1000 appears superior to dextran-coated charcoal, since it does not bind protein. Therefore it can be adequately used for the preparation of fatty acid-free serum albumin (chapter 9). The assay of fatty acid-binding capacities with Lipidex 1000 is also much more specific and accurate than with Sephadex G-25 (Ockner & Manning, 1974) or dextran-coated charcoal. The method appears particularly suitable for following purification of fatty acid-binding proteins (chapter 11) and for studying detergent binding to membrane proteins more rapidly than with gel-filtration or equilibrium-dialysis techniques.

During the course of these studies, several other investigators have presented alternative procedures for the assay of fatty acid binding by proteins. Morrow & Martin (1983) described a method, which in principal is

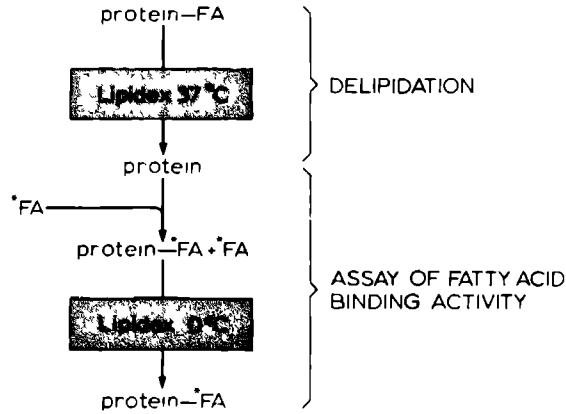



Fig. 8.6. Schematic presentation of the use of Lipidex 1000 at 37°C for the delipidation of protein samples and at 0°C for the separation of protein-bound and unbound fatty acids. FA, endogenous fatty acid; *FA, labeled fatty acid.

comparable to ours, but uses dextran-gelatin-coated charcoal for the separation of unbound and protein-bound fatty acids. As demonstrated in this chapter, however, dextran-coated charcoal is disadvantageous over Lipidex, because of its binding properties for protein (Table 8.1; Fig. 8.3). This protein binding also explains the observed loss of assay sensitivity and linearity when the binding proteins were exposed to unsedimented charcoal for periods longer than 3-4 min (Morrow & Martin, 1983) (cf. Fig. 8.1). Although these workers found a similar palmitate binding by bovine serum albumin, when compared to our data with dextran-coated charcoal, their assay appears less sensitive as they apply at least 10 µg of albumin (cf. Fig. 8.2).

Smith *et al.* (1983) presented an electrophoretic technique and Keuper *et al.* (1983) a modified equilibrium-dialysis method, but both procedures appear more complicated and time-consuming than our assay. Fournier *et al.* (1983a,b) studied the binding capacity of pig heart FABP for spin-labeled fatty acid (12-doxylstearate) by electron spin resonance spectroscopy. The spectral changes induced by binding of the ligand were found to be of sufficient amplitude to make detailed investigations possible. Although promi-

sing, this technique appears not suitable for the routine assay of fatty acid binding by proteins.

With our method we found an apparent $K_d$ for palmitate binding by liver cytosolic proteins, which is identical to that observed for palmitate with equilibrium-dialysis studies (Ketterer *et al.*, 1976), but 3-fold lower than reported for oleate on the basis of coelution with cytosolic proteins from a gel-filtration column (Mishkin *et al.*, 1972). Our value for $K_d$ is also of comparable magnitude to that reported for the $M_r$ 16,000 sterol carrier protein from rat liver (Daum & Dempsey, 1980), which protein might be identical to the fatty acid-binding protein (Dempsey *et al.*, 1981; Billheimer & Gaylor, 1980) (cf. chapters 10 and 11). Palmitoyl-CoA was found to bind to low-$M_r$ cytosolic proteins with a much higher affinity than palmitate (Mishkin & Turcotte, 1974; Ketterer *et al.*, 1976). With equilibrium-dialysis the total fatty acid-binding capacity of the liver cytosol was found to be only 0.1 pmol/µg protein (Ketterer *et al.*, 1976).

If we assume that the fatty acid binding by the cytosolic proteins can entirely be attributed to the $M_r$ 12,000 fatty acid-binding protein (FABP) and that the number of fatty acid-binding sites on this protein is one, it appears from the maximal fatty acid binding that the cytosolic FABP concentration is 4.39 × 12 = 53 µg/mg protein. By quantitative radial immunodiffusion, Ockner *et al.* (1982) recently found the rat liver cytosol to contain 40-50 µg FABP/mg protein. Comparison of these values establishes that FABP possesses only one binding site for long-chain fatty acids per protein molecule (cf. chapter 11). This calculation is in accordance with the observations that the number of endogenous fatty acids associated with one molecule of the protein never exceeds one (Burnett *et al.*, 1979; Rüstow *et al.*, 1982).

## 8.5. SUMMARY

Protein-bound and unbound fatty acids can be efficiently separated at 0°C using Lipidex 1000, a hydrophobic column-packing material, just as this substance can separate protein-bound and unbound steroids (Dahlberg *et al.*, 1980). Protein-bound fatty acids are also removed by Lipidex 1000 when treatment is performed at 37°C.

Lipidex 1000 does not exhibit binding properties for soluble proteins at 0 and 37°C, in contrast to dextran-coated charcoal. It appears useful

for the delipidation of protein samples at 37°C and for a radiochemical assay of fatty acid binding by µg-amounts of protein at 0°C. With this assay we have obtained results on palmitate binding to serum albumin similar to those reported on the basis of equilibrium-dialysis.

Delipidated proteins from dealbuminized rat liver cytosol maximally bind about 4 nmol palmitate/mg protein with a $K_d$ of 1 µM.

# CHAPTER 9

# REMOVAL OF FATTY ACIDS FROM SERUM ALBUMIN BY LIPIDEX 1000 CHROMATOGRAPHY*

## 9.1. INTRODUCTION

Defatting of serum albumin is usually performed by treatment with activated charcoal at pH 3 and 0°C, according to the method of Chen (1967). Although by this procedure the protein remains native, as judged by a number of criteria (Chen, 1967), the treatment at low pH leads to an artificial broadening of the microheterogeneity of the serum albumins, probably as a result of some deamidation of the protein (Sogami & Foster, 1968). Certain irreversible changes in the albumin molecule were also observed after acid-charcoal treatment (Steinhardt *et al.*, 1972). Furthermore, at pH around 3 a dimerization of albumin was reported to occur (Williams & Foster, 1960).

In the previous chapter we have shown that Lipidex 1000 (a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25) can be used for the temperature-dependent removal of fatty acids from a protein-fatty acid complex and for the detection and assay of fatty acid-binding proteins. In this chapter we report that serum albumin can be conveniently freed from fatty acids by a single passage through a column of Lipidex 1000 at 37°C.

The removal of fatty acids was studied by determining the fatty acid content of the albumin preparations before and after treatment. To investigate fatty acid binding by the protein, we used the described radiochemical assay procedure in which Lipidex is applied at 0°C (chapter 8).

* Adapted from Glatz & Veerkamp (1983b).

*Treatment of Lipidex 1000*

Lipidex 1000 was washed free of methanol in a column by elution with 10 bed volumes of 10 mM K-phosphate (pH 7.4), containing 0.01% $NaN_3$. For fatty acid binding experiments the gel was transferred to a glass vial and stored at 4°C as a Lipidex-buffer suspension (1:1, v/v) containing about 100 mg dry Lipidex/ml buffer.

*Delipidation of serum albumin*

Serum albumin was defatted as described by Chen (1967) or by Lipidex 1000 chromatography. For the latter 0.5 g of serum albumin was dissolved in 5 ml of distilled water and applied to a column of Lipidex (3.0 × 15 cm) equilibrated with water at 37°C (thermostated column; flow rate, 150 ml/h). Elution of the protein was performed with water, and the protein was recovered in the void volume. The protein content of the eluant was determined as described in section 2.3.

The column was reused after thorough washing with methanol (chapter 8). After defatting, the serum albumin preparations were dialyzed against physiological saline (60 h) and distilled water (6 h) in order to remove the relatively large amounts of citrate, present in commercial albumin (Lopes-Cardozo & Van den Bergh, 1972; Hanson & Ballard, 1968). The preparations were neutralized with 0.2 M KOH, if necessary, and lyophilized.

*Determination of fatty acid content and of fatty acid binding*

For determination of the fatty acid content, portions of 100 mg of the preparations were dissolved in 1 ml of distilled water and subjected to lipid extraction according to Dole & Meinertz (section 2.3). Fatty acids were assayed radiochemically using $^{63}Ni$ as tracer, as described in section 2.3 . Fatty acid content was calculated on the basis of a molecular weight of bovine serum albumin of 68,000.

Samples of 2 or 5 µg albumin were assayed for fatty acid binding according to the procedure described in chapter 8. The binding of [1-$^{14}$C]-palmitate was measured at 0.1, 1 and 4 µM, and is expressed as pmol/µg protein.

*Table 9.1. Removal of fatty acids from bovine serum albumin by acid-charcoal treatment or Lipidex chromatography.*

| Preparation | Fatty acid content (mol/mol protein) | Palmitate binding (pmol/μg protein) | | |
|---|---|---|---|---|
| | | 0.1 μM palmitate | 1 μM palmitate | 4 μM palmitate |
| Fraction V, Sigma Lot 80 F-0508 (untreated) | 0.496 ± 0.070 | 14.3 ± 1.0 | 35.5 ± 2.2 | 75.9 ± 4.3 |
| After charcoal treatment at pH 3 and 0°C | 0.036 ± 0.009 | 14.4 ± 1.3 | 35.0 ± 3.1 | 79.7 ± 4.6 |
| After Lipidex 1000 chromatography at 37°C | 0.049 ± 0.010 | 15.4 ± 0.7 | 39.4 ± 2.1 | 89.3 ± 10.6 |

Fatty acids were extracted from the protein samples and assayed radiochemically with the use of $^{63}Ni$ as tracer. The binding of [1-$^{14}$C]palmitate was measured at 0.1 μM with 2 μg albumin and at 1 and 4 μM with 5 μg albumin. Values represent means ± S.D. of 4 experiments.

## 9.3. RESULTS AND DISCUSSION

Untreated serum albumin was found to contain about 0.5 mol fatty acid per mol protein (Table 9.1). This content is comparable to that reported previously for various commercial albumin preparations (Chen, 1967) [1]. After charcoal treatment and also after Lipidex 1000 chromatography at least 90% of the fatty acids were removed (Table 9.1), indicating that both methods are suitable for defatting serum albumin. Lipidex 1000 chromatography at 37°C has, however, some advantages over the acid-charcoal treatment procedure. The former procedure is milder and more rapid, since the harmful low pH step and the centrifugation step are avoided. Furthermore, with charcoal

[1] Bovine serum albumin obtained from Merck (Darmstadt, F.R.G.; Lot 0072449) was found to contain 3.14 ± 0.43 mol fatty acid per mol protein (mean ± S.D., 3 determinations). We have not attempted to delipidate this preparation by either procedure.

treatment protein recovery was in our hands 75-80%, as also reported by Soagami & Foster (1968). With Lipidex 1000 chromatography the recovery amounted to 94 ± 3% (mean ± S.D. of 4 determinations). A comparable protein recovery was obtained after chromatography on Lipidex of *N*-[$^{14}$C]methylated bovine serum albumin (section 8.3).

The maximal fatty acid-binding capacity of Lipidex was estimated by chromatography at 37°C of a complex of [1-$^{14}$C]palmitate to bovine serum albumin (molar ratio 1:1) and found to be 40-50 nmol/ml packed gel. Other lipid impurities, such as phospholipids and cholesterol, present in most commercially available albumin preparations (Fainaru *et al.*, 1981), will also be removed by Lipidex chromatography, since the gel shows high affinity for lipids and steroids (Dahlberg *et al.*, 1980).

The ability of serum albumin to bind fatty acids was determined with the assay procedure described in chapter 8. At the palmitate concentrations tested (0.1, 1 and 4 μM) the fatty acid binding did not change significantly after charcoal treatment nor after Lipidex chromatography, when compared to the binding by untreated albumin (Table 9.1). Also after a second passage through the Lipidex column identical fatty acid-binding values were obtained at the three palmitate concentrations (data not shown), indicating that the binding capacity of the albumin preparation was not influenced by the chromatographic procedure. The values obtained at 4 μM palmitate correspond to a palmitate:albumin molar ratio of 5-6, which is comparable to the ratio measured with equilibrium-dialysis (Spector, 1975).

A lower fatty acid binding with the untreated albumin preparation, especially at a lower palmitate concentration, would be expected due to competition of endogenous fatty acids. With rat liver cytosolic proteins fatty acid binding was significantly lower before delipidation, when assayed at 0.2 μM, but not at 1 μM [1-$^{14}$C]palmitate (Table 8.4, page 129). However, this competition can not be detected with the commercial albumin preparation, since the amount of endogenous fatty acids present (0.5 mol/mol protein, Table 9.1) was relatively low. Also, due to its high fatty acid-binding capacity, the amount of albumin which can be applied in the assay is restricted in relation to the free fatty acid concentration in order to maintain a linear dependence in the assay.

The results show that fatty acids can be adequately removed from serum albumin preparations by chromatography at 37°C on a column of Lipidex 1000 without loss of protein and with maintenance of the fatty acid-binding

capacity. If desired, the adsorbed fatty acids can be eluted with methanol (Dahlberg *et al.*, 1980; chapter 8) for further analysis. The method also appears to be useful for delipidation of other lipid-binding or lipid-transferring proteins and membrane proteins, and is of value particularly when the availability of protein is limited.

Recently, Nakano *et al.* (1983) described the use of activated carbon beads for the chromatographic removal of residual fatty acids from albumin. Although it was shown that albumin is not retained by the beads, a low pH was still necessary for the complete removal of the fatty acids.

## 9.4. SUMMARY

Fatty acids can be effectively removed from serum albumin preparations by a single passage through a column of Lipidex 1000 at 37°C. The procedure is easier and milder and shows a better (nearly quantitative) recovery of protein than acid-charcoal treatment. The ability for fatty acid binding by the protein is not affected by either procedure.

# CHAPTER 10

# DIURNAL VARIATION OF CYTOSOLIC FATTY ACID-BINDING PROTEIN CONTENT AND OF PALMITATE OXIDATION IN RAT LIVER AND HEART, AND THE EFFECT OF CHOLESTYRAMINE FEEDING*

## 10.1. INTRODUCTION

Fatty acid-binding protein (FABP), which is found in the cytosol of many eucaryotic cells, exhibits a high affinity for long-chain fatty acids and their corresponding coenzyme A thioesters and therefore is considered to function in the intracellular fatty acid utilization (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). The rather high concentration of this $M_r$ 12,000 protein in liver (Ockner *et al.*, 1982) suggests that it may also serve for storage of fatty acids and their derivatives. In liver and intestine, modulations of the rates of fatty acid uptake or utilization, in response to nutritional, hormonal and pharmacological manipulations, correlated with changes in the cytosolic content of FABP (Mishkin *et al.*, 1975; Fleischner *et al.*, 1975; Renaud *et al.*, 1978; Burnett *et al.*, 1979; Ockner *et al.*, 1979, 1980). There is a growing amount of evidence that FABP is similar, if not identical, to sterol carrier protein$_1$ ($SCP_1$) in physical and molecular properties and in tissue distribution (Billheimer & Gaylor, 1980; Dempsey *et al.*, 1981). $SCP_1$ is required for activation of membrane-bound enzymes catalyzing cholesterol synthesis and metabolism to bile acids and steroid hormones (Dempsey, 1974; Srikantaiah *et al.*, 1976; Billheimer & Gaylor, 1980; Song & Dempsey, 1981). Recently, Dempsey *et al.* (1980) reported that in liver $SCP_1$ undergoes a significant diurnal variation corresponding in time to that of major enzymes in lipid metabolism, such as hydroxymethylglutaryl-CoA reductase (Shapiro & Rodwell, 1969; Rodwell *et al.*, 1976) and methyl sterol oxidase (Spence & Gaylor, 1977). The hepatic $SCP_1$ content varied from 1 to 10% of the total cytosolic protein during a 24 h light-dark cycle

*Adapted from Glatz *et al.* (1983b) and Kempen *et al.* (1983b).

and is probably regulated at the level of translation of $SCP_1$-mRNA (McGuire *et al.*, 1983).

In this chapter we show that rat heart cytosol contains a previously unrecognized fatty acid-binding capacity, which is similar to that of liver cytosol, and furthermore that this capacity exhibits a diurnal rhythm in both heart and liver, being about 2-fold higher at the mid-dark than at the mid-light phase of the light cycle. In concert, the palmitate oxidation capacity and the citrate synthase activity were significantly elevated during the dark period in both tissues. The results provide further evidence for a potential role of FABP in cellular fatty acid utilization and for its similarity with $SCP_1$.

In addition, we also studied the effect of cholestyramine feeding on the fatty acid-binding capacity of the liver cytosol and its diurnal variation. The bile salt sequestrant cholestyramine not only increases the rate of hepatic cholesterol and bile acid synthesis (e.g. Kempen *et al.*, 1982, 1983a), but also increases the plasma level and the hepatic synthesis of triacylglycerols and of very low density lipoproteins (Nestel & Grundy, 1976; Witztum *et al.*, 1976; Angelin *et al.*, 1978; Adler *et al.*, 1978; Kempen *et al.*, 1982). It appeared plausible to us that this effect of cholestyramine feeding is mediated by a rise in the cytosolic content of FABP, because of the above-mentioned similarity of FABP with $SCP_1$, and since FABP stimulates hepatic synthesis of triacylglycerols in cell-free systems (O'Doherty & Kuksis, 1975; Burnett *et al.*, 1979; Iritani *et al.*, 1982).

## 10.2. MATERIALS AND METHODS

### *Preparation of cytosol*

Male albino Wistar rats, weighing 180-220 g, were mainained on a cycle of alternating 12-h periods of light and darkness and were sacrificed at the midpoint of the light or of the dark period. The rats were fed ad libitum on a control or cholestyramine-containing diet, as described in section 2.1. Rat liver and heart cytosol were prepared as described in section 2.2.

The amount of residual albumin present in the liver cytosol preparations was found to be negligible, as judged by cross-over electrophoresis against rabbit anti-rat albumin antiserum. The heart cytosol preparations, however, initially still contained some albumin, which was removed by passage at $4^{\circ}C$ through an affinity column of the immunoglobulin G fraction of

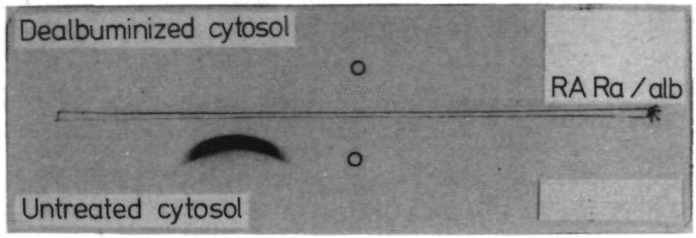



Fig. 10.1. Immunoelectrophoresis of cytosolic proteins from rat heart, before and after dealbuminization. About 1 µg of protein was placed in the center well and subjected to electrophoresis (pH 8.2). Rabbit anti-rat albumin antiserum (RARa/alb) was then placed in the trough and allowed to diffuse in the direction of the separated proteins. The precipitin line indicates the presence of rat albumin. The results are shown of a representative experiment.

rabbit anti-rat albumin antiserum bound to Protein A-Sepharose CL-4B. The complete removal of albumin after this treatment was confirmed by both cross-over electrophoresis and immunoelectrophoresis (Fig. 10.1).

For delipidation of the cytosolic proteins, portions of about 30 mg protein were subjected to chromatography on a column of Lipidex (1 × 15 cm), equilibrated with 10 mM K-phosphate buffer (pH 7.4) at 37°C. Elution was performed with the same buffer (flow rate 20 ml/h). All protein was present in the void volume.

*Assays*

Fatty acid binding by cytosolic protein was assayed according to the method described in chapter 8. Protein samples were incubated in 10 mM K-phosphate buffer (pH 7.4) with various concentrations of [1-$^{14}$C]palmitate. Fatty acid binding is expressed as pmol/µg protein. The binding curves were analyzed by the method of Scatchard (1949). The concentration of free fatty acids was calculated from the initial concentration and the amount of protein-bound fatty acid. No correction was applied for fatty acid binding to the polyethylene vial (cf. section 8.3).

Palmitate oxidation was measured with 25 µl (heart) or 50 µl (liver) whole homogenate (5%, w/v) as described in section 3.2. Assays of protein and of citrate synthase activity are described in section 2.3.

*Palmitate binding by cytosolic protein*

The binding of palmitate increased proportional to the amount of protein at both 0.2 and 1 μM [1-$^{14}$C]palmitate (up to the binding of about 50-60% of the available palmitate; cf. Fig. 8.2), only when delipidated protein samples were used (Fig. 10.2). With cytosol preparations that had not been delipidated a non-linear relationship was observed, and at a higher amount of protein the palmitate binding was underestimated to an increasing extent. This underestimation is due to label dilution by endogenous fatty acids and possibly also to competition by endogenous ligands, other than fatty acids, which were also found to be associated with cytoso-

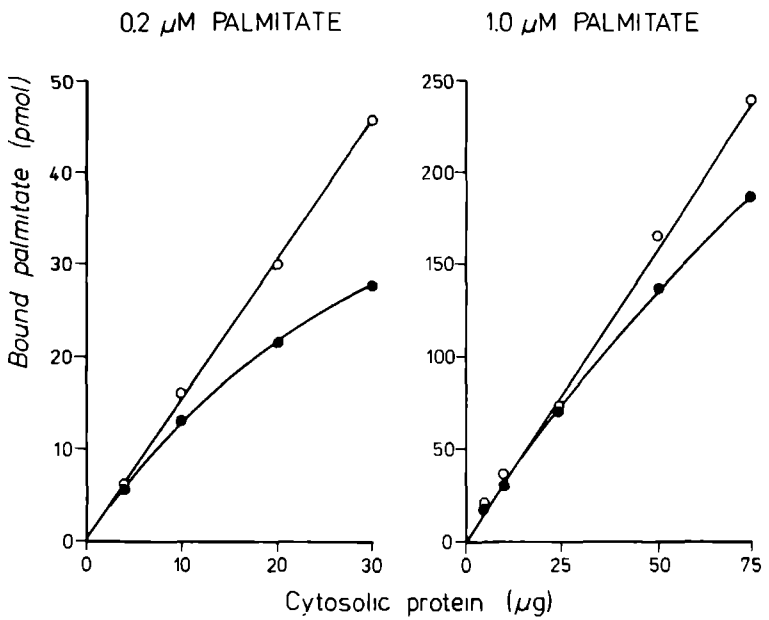



Fig. 10.2. Effect of delipidation on the dependence of palmitate binding on the amount of cytosolic protein. Various amounts of non-delipidated (●) and of delipidated (o) liver cytosolic protein were assayed for palmitate binding in the presence of 0.2 μM (left) and 1.0 μM (right) [1-$^{14}$C]palmitate. The results are shown of one representative experiment out of three, using the liver cytosol obtained from rats sacrificed at the mid-dark phase of the light cycle. With cytosol preparations from rat heart similar results were obtained (data not shown).

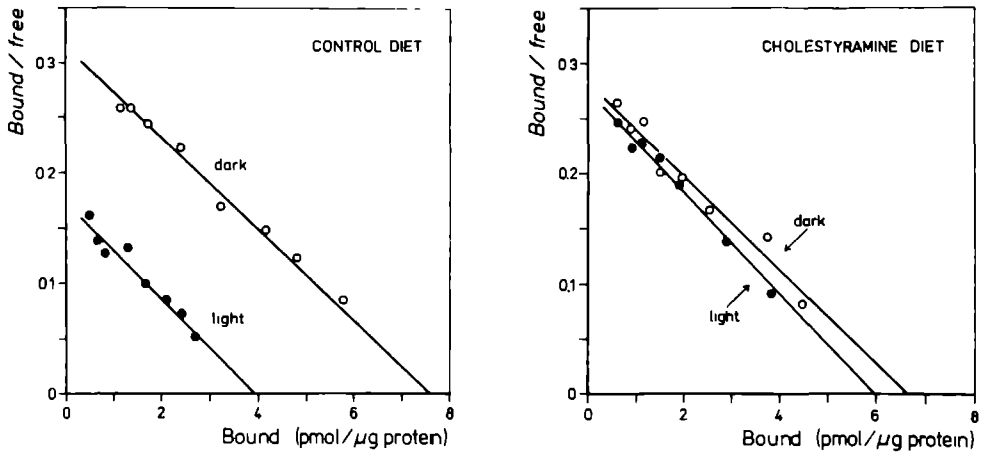



Fig. 10.3. Scatchard analysis of the binding of palmitate by delipidated liver cytosol from control (left) and cholestyramine-fed (right) rats sacrificed at the mid-dark (o) or at the mid-light (•) phase of the light cycle. Cytosolic protein samples were incubated with 0.1 - 3 µM [1-$^{14}$C]palmitate an after equilibration protein-bound and unbound palmitate were separated by the use of Lipidex at 0°C. The results are shown of representative experiments, in which 20 µg (control diet) and 18 µg (cholestyramine diet) of protein were used per assay.

lic FABP (Burnett *et al.*, 1979; Ockner *et al.*, 1982; Rüstow *et al.*, 1982). This competition is more pronounced at higher protein and lower palmitate concentrations (Fig. 10.2). Palmitate binding did not decrease after storage of the protein samples at -20°C (up to 6 months).

With the delipidated cytosol of liver and heart, obtained from rats sacrificed at the mid-dark or at the mid-light phase of the light cycle, we studied the binding of palmitate by cytosolic protein as a function of the total palmitate concentration. Scatchard plot analysis of the binding isotherms revealed the presence of a single class of saturable binding sites on the cytosolic proteins from both tissues (Figs. 10.3 and 10.4). At the mid-dark phase both the liver and heart cytosol contain a significantly higher capacity for palmitate binding per µg protein than at the mid-light period (Figs. 10.3 and 10.4; Table 10.1). With both types of cytosol preparations, however, half-maximal saturation is reached at the same palmitate concentration (1 and 0.8 µM for rat liver and heart, respectively). These findings suggest that at the mid-dark phase the liver and heart cytosol

*Table 10.1. Diurnal variation of palmitate binding by cytosolic protein and of endogenous fatty acid content of the cytosol in rat liver and heart.*

| Tissue | Diet | Condition | | Palmitate binding | | Endogenous free fatty acid content |
|---|---|---|---|---|---|---|
| | | | | $B_{max}$ (pmol/μg protein) | Apparent $K_d$ (μM) | (pmol/μg protein) |
| Liver | Control | Light | (3) | 3.92 ± 0.38 | 1.04 ± 0.15 | 1.47 ± 0.25 |
| | | Dark | (3) | 7.20 ± 0.39 [a] | 0.98 ± 0.14 | 1.68 ± 0.53 |
| | Cholesty-ramine | Light | (7) | 6.03 ± 0.67 [c] | 1.06 ± 0.22 | 1.81 ± 0.23 |
| | | Dark | (4) | 6.79 ± 0.48 | 0.90 ± 0.10 | 1.37 ± 0.53 |
| Heart | Control | Light | (4) | 3.49 ± 0.39 | 0.83 ± 0.11 | 1.91 ± 0.22 |
| | | Dark | (3) | 8.02 ± 0.70 [b] | 0.80 ± 0.25 | 2.87 ± 0.18 [a] |

Data on palmitate binding are derived from Scatchard analysis of individual binding isotherms, as shown in Fig. 10.3 (liver) and Fig. 10.4 (heart). The amounts of delipidated protein used per assay were 15-25 μg.
For determination of the endogenous free fatty acid content, samples of 20-40 mg cytosolic protein were subjected to lipid extraction according to the method of Dole & Meinertz (1960). Phospholipids then were removed from the free fatty acids by binding to silicic acid. Thereafter the fatty acids were assayed radiochemically using $^{63}Ni$ as tracer, as described in section 2.3. For comparison, the fatty acid content is also expressed as pmol/μg protein.
All values represent means ± S.D. of the number of separate preparations given within parentheses. Values are compared by the unpaired *t*-test.

[a] $P < 0.01$, dark *versus* light

[b] $P < 0.001$, dark *versus* light

[c] $P < 0.001$, cholestyramine diet *versus* control diet (light period).

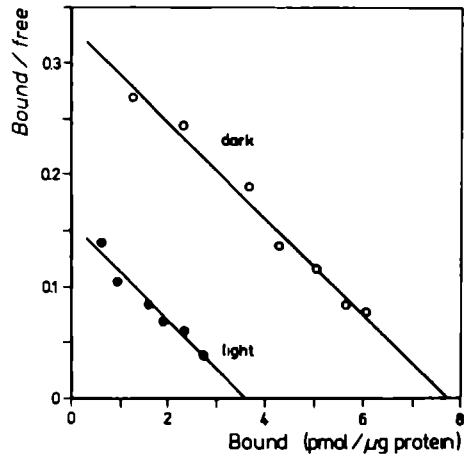



Fig. 10.4. Scatchard analysis of the binding of palmitate by the dealbuminized and delipidated heart cytosol from rats sacrificed at the mid-dark (o) or at the mid-light (•) phase of the light cycle. The results are shown of representative experiments, using 18 µg of protein per assay. For further experimental details, see the legend to Fig. 10.3.

contain 1.8 - and 2.3 -fold more binding sites, respectively, but on the same molecular species (Table 10.1). Since at the mid-dark phase of the light cycle the rat liver weight is about 1.5 -fold higher than at the mid-light phase (Seifert, 1980), the total amount of fatty acid-binding sites expressed per whole liver even shows a 2.7 -fold diurnal variation.

When the cytosolic proteins were fractionated by chromatography on Sephadex G-75, the major fatty acid-binding fraction eluted at the same position with all preparations examined (Fig. 10.5). This fraction corresponds to $M_r$ 12,000-14,000 and contains FABP (Fournier *et al.*, 1978; Ockner *et al.*, 1982). The specific palmitate binding activity of this fraction was also 1.5 - 2 -fold higher in the cytosol preparations from rats sacrificed at the mid-dark compared to the mid-light phase, amounting to 28-36 and 20-23 pmol/µg protein, respectively, for the liver cytosol, and to 10-12 and 6-8 pmol/µg protein, respectively, for the heart cytosol. The specific binding capacities are under both conditions lower in heart than in liver, due to the relatively higher content of low-$M_r$ proteins (e.g. myoglobin) of heart muscle (Fig. 10.5). The small fatty acid-binding fraction of $M_r$ 1500-2000, which contains a fatty acid-binding peptide (Suzue & Marcel,

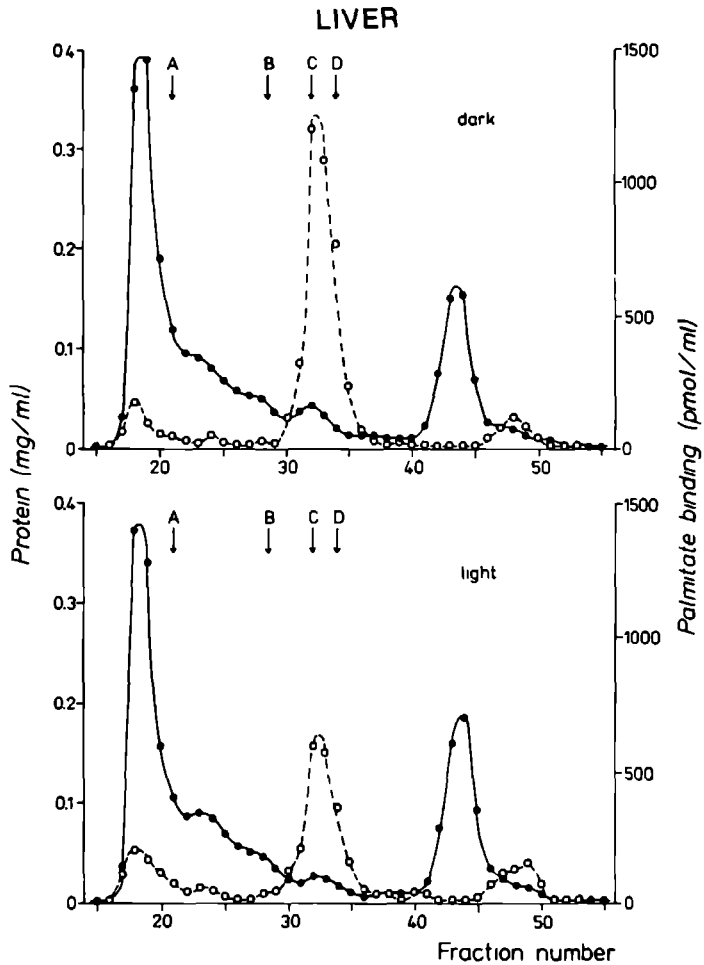
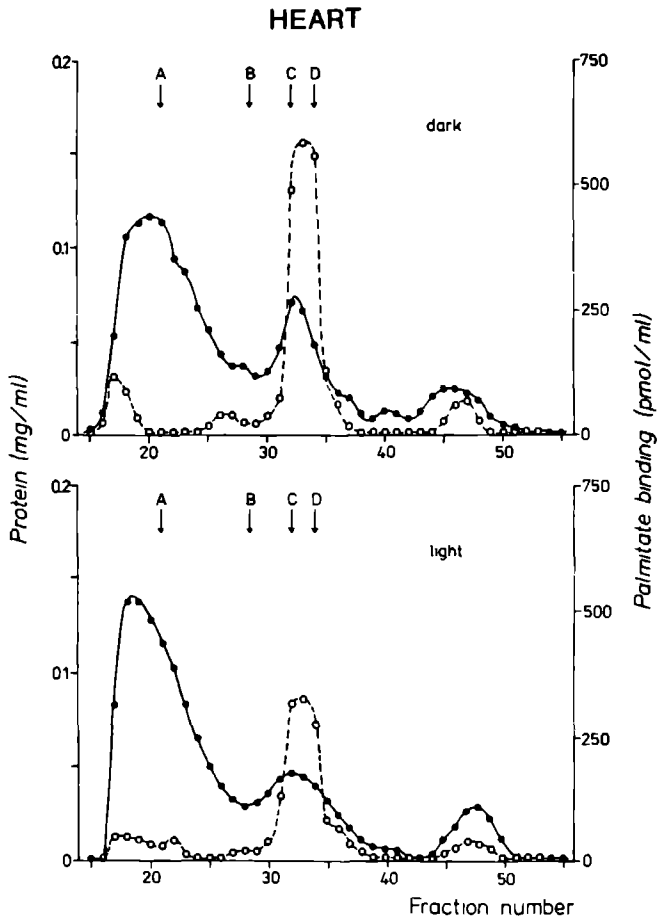



Fig. 10.5. Gel-filtration on Sephadex G-75 (1.6 x 85 cm) of 10 and 5 mg delipidated cytosolic protein from liver (this page) and from heart (facing page), respectively, of rats sacrificed at the mid-dark or at the mid-light phase of the light cycle.
Elution was performed at 4°C with 10 mM K-phosphate buffer (pH 7.4). The flow rate was 20 ml/h and the fraction volume 3.7 ml.
All fractions were assayed for protein (●) and for palmitate binding (o). For the latter 50-200 -µl samples and 1 µM [1-$^{14}$C]-palmitate were used.
Arrows indicate the elution positions of calibration proteins: A, rat serum albumin; B, α-chymotrypsinogen; C, horse skeletal muscle myoglobin and D, horse heart cytochrome *c*.
The recoveries of protein as well as of fatty acid-binding activity from the column were 85-95%. The results are shown of one representative experiment out of three.




1975; Rüstow *et al.*, 1978, 1979), did not show a significant diurnal rhythm (Fig. 10.5). These results show that the higher palmitate binding capacity per µg of protein of both the rat liver and heart cytosol during the dark relative to the light period can be attributed to a higher content of FABP or to an increased availability of the fatty acid-binding site(s) on this protein. The former possibility is most likely since modulation of the hepatic content of FABP is known to occur in several conditions involving nutritional, hormonal and pharmacological manipulations (Mishkin *et al.*, 1975; Fleischner *et al.*, 1975; Renaud *et al.*, 1978; Burnett *et al.*, 1979; Ockner *et al.*, 1979, 1980), whereas no evidence is available on the regulation

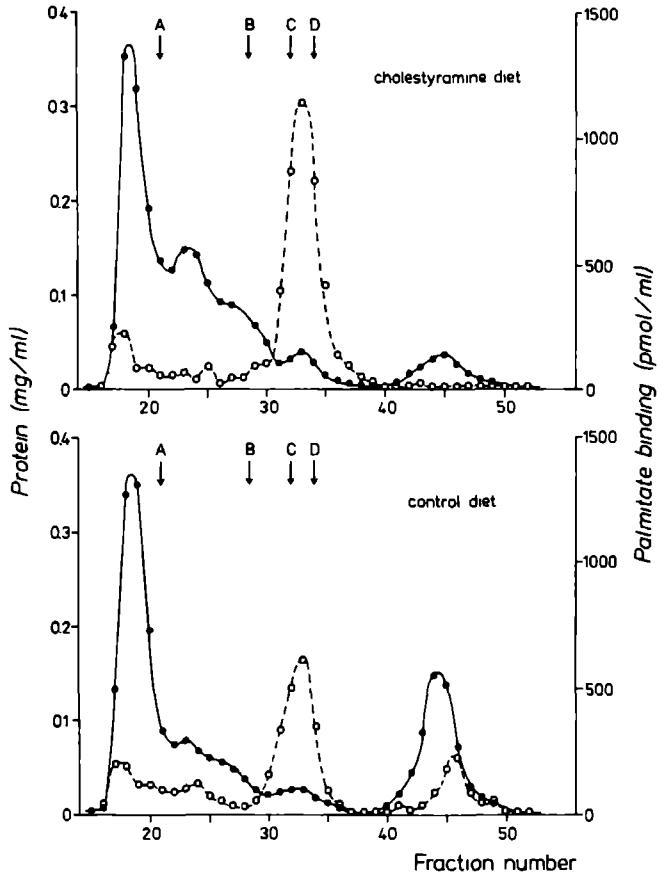



Fig. 10.6. Gel-filtration on Sephadex G-75 of 10 mg liver cytosolic protein from control and cholestyramine-fed rats sacrificed at the mid-light phase of the light cycle.
All fractions were assayed for protein (●) and for palmitate binding (o). The results are representative for three independent experiments with each group of rats.
See the legend to Fig. 10.5 (page 144) for further details.

of the availability of the fatty acid-binding site(s) of FABP.

By comparison of the maximal fatty acid binding by rat liver cytosolic proteins with the cytosolic FABP concentration as found by Ockner *et al.* (1982) on basis of quantitative radial immunodiffusion studies, we established (section 8.4) that FABP possesses only one fatty acid-binding site per protein molecule (cf. section 11.3). As FABP accounts for 75-85% of the fatty acid binding of our cytosol preparations (Fig. 10.5) and has a $M_r$ of 12,000 (Ockner *et al.*, 1982), the cytosolic FABP concentration can be estimated from the maximal fatty acid binding (Table 10.1). It appears that in liver the cytosolic FABP concentration amounts to about 40 and 70 µg/mg protein at the mid-light and the mid-dark phase of the light cycle, respectively, and in heart to about 35 and 80 µg/mg protein, respectively.

*Effect of cholestyramine feeding*

Cholestyramine feeding increased the palmitate binding capacity of the delipidated liver cytosol at the mid-light phase about 1.5-fold (Fig. 10.3; Table 10.1). Sephadex G-75 chromatography of the cytosolic proteins revealed that this increase can also be attributed to an elevation of the content or binding activity of FABP (Fig. 10.6). Cholestyramine feeding did, however, not further increase the palmitate binding capacity of the liver cytosol at the mid-dark phase, so that with this diet there is no significant diurnal variation of hepatic FABP activity. It is plausible that the levels of palmitate binding capacity reached during the dark period or after cholestyramine feeding represent a maximum.

*Endogenous fatty acid content*

Since endogenous fatty acids are considered to be mainly present associated with FABP, we also examined the possible diurnal variation of the fatty acid content of liver and heart cytosol. In liver the cytosolic fatty acid content did not differ between the dark and the light period, nor between cholestyramine-fed and control animals, and amounted to 1.4 - 1.8 nmol/mg protein (Table 10.1). Burnett *et al.* (1979) also reported a fatty acid content of 1.4 nmol/mg protein for liver cytosol of rats sacrificed during the light period. The cytosolic fatty acid content of heart was at the mid-light phase of the cycle comparable to that of liver, but significantly higher at the mid-dark phase (Table 10.1). Comparison of these values with the palmitate binding capacity of the various prepara-

tions (Table 10.1) reveals that under all conditions examined the degree of occupancy of the available binding sites did never exceed 50%. In liver, the degree of occupancy drops to about 20% at the mid-dark period of the light cycle.

The yield of cytosolic protein amounted to 25-35 and 15-20 mg/g wet weight tissue for rat liver and heart, respectively, so that at the mid-light phase the endogenous fatty acid content is about 40-55 and 30-40 nmol /g wet weight tissue, respectively. For heart this value is of the same order of magnitude as the nonesterified fatty acid concentration reported by others (Garland & Randle, 1963; Kramer & Hulan, 1978; Van der Vusse *et al.*, 1980).

*Table 10.2. Diurnal variation of palmitate oxidation and citrate synthase activity in rat liver and heart.*

| Tissue | Condition | | Palmitate oxidation | | Citrate synthase activity |
|---|---|---|---|---|---|
| | | | /g wet wt | /unit citrate synthase | unit/g wet wt |
| Liver | Light | (5) | 366 ± 28 | 31.7 ± 3.1 | 11.6 ± 0.4 |
| | Dark | (7) | 508 ± 39 [a] | 32.5 ± 1.9 | 15.6 ± 1.1 [a] |
| Heart | Light | (7) | 733 ± 47 | 10.04 ± 1.03 | 71.6 ± 4.9 |
| | Dark | (7) | 824 ± 31 [b] | 8.88 ± 0.63 | 93.0 ± 5.9 [a] |

Oxidation rates were measured with 120 μM [1-$^{14}$C]palmitate bound to albumin (molar ratio 5:1), and were calculated from the sum of the production of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products. Values represent means ± S.D. of the number of preparations given within parentheses. Values are compared by the unpaired *t*-test.

[a] $P < 0.001$, dark *versus* light

[b] $P < 0.01$, dark *versus* light

*Palmitate oxidation and citrate synthase activity*

With homogenates of rat liver and heart palmitate oxidation proceeded proportional with time of incubation and amount of tissue material. The oxidation was measured at concentrations of substrate, coenzymes and cofactors that gave maximal oxidation rates (Van Hinsbergh *et al.*, 1978a; section 3.3). In both liver and heart the palmitate oxidation capacity, expressed per g of tissue, was significantly increased at the mid-dark compared to the mid-light phase of the light cycle (Table 10.2). The citrate synthase activity showed a similar increase in both tissues, so that when the oxidation rates are expressed relative to this mitochondrial marker enzyme, hardly any diurnal changes are observed (Table 10.2). These results indicate that the diurnal fluctuations of the palmitate oxidation capacity are related to fluctuations of total mitochondrial activity in both tissues.

## 10.4. DISCUSSION

Both immunochemical and non-immunochemical methods have been applied for the estimation of the total fatty acid-binding capacity of the cytosol or the quantitation of FABP (cf. Ockner *et al.*, 1980, 1982). The former procedure requires the availability of precipitating antibodies reactive to all forms of FABP that may be present in the cytosol. There is now evidence, however, for molecular heterogeneity of FABP in liver (Ketterer *et al.*, 1976; Trulzsch & Arias, 1981; Morrow & Martin, 1980, 1983). Although some forms of FABP cross-reacted with monospecific antibodies raised against them (Trulzsch & Arias, 1981), at least one hepatic form was found to be not highly antigenic (Morrow & Martin, 1983). The various forms may furthermore exhibit a different affinity for long-chain fatty acids (cf. Ketterer *et al.*, 1976; Trulzsch & Arias, 1981). A non-immunochemical method, such as the assay used in this study, may be of more physiological interest, since it provides a functional quantitation of the cytosolic fatty acid-binding activity.

Few laboratories have to date reported on FABP of heart cytosol (Mishkin *et al.*, 1972; Fournier *et al.*, 1978, 1983a,b; Rüstow *et al.*, 1982), although its presence in heart muscle was established already in the first report on FABP (Ockner *et al.*, 1972). Quantitative data on cardiac FABP were only presented in two papers (Mishkin *et al.*, 1972; Rüstow *et al.*, 1982). Miskin *et al.* (1972) reported that the relative amount of oleic acid that

coelutes with the FABP-containing fraction upon gel-filtration of cytosolic proteins was for rat heart about 30% of that of rat liver. This comparison is, however, of only limited value since the preparations may have contained different amounts of albumin and, as delipidation was omitted, of endogenous ligands, and since the applied assay method is rather inaccurate due to the high affinity of the fatty acids for the gel itself. Rüstow *et al.* (1982) used an antibody raised against purified rat liver FABP and estimated that rat heart cytosol contains a 10-fold lower amount of FABP than liver cytosol. The former amount has likely been underestimated since the physical and molecular properties of FABP from rat heart and liver are different (chapter 11), and immunochemical cross-reactivity might therefore be low. Myoglobin has also been suggested to act as a fatty acid-binding protein in muscle (Gloster & Harris, 1977; Harris *et al.*, 1980), but this appears unlikely because of its low fatty acid-binding affinity (Table 8.2, page 124) and the fact that upon ion-exchange chromatography of rat heart cytosol, FABP ($M_r$ 12,000) is completely separated from myoglobin (Fournier *et al.*, 1978; chapter 11). Using albumin-free and delipidated rat heart cytosol preparations we found with our assay a similar palmitate binding capacity as in liver cytosol in both phases of the light cycle. As the palmitate binding could almost entirely be attributed to proteins of $M_r$ 12,000-14,000, it was calculated that FABP will make up about 4-8% of the rat heart soluble proteins. This abundance of FABP as well as its known properties support the view that also in heart muscle this protein plays an important regulatory role in lipid metabolism. In heart FABP may act as a protector for the detrimental effects of high intracellular levels of long-chain fatty acids and fatty acyl-CoA thioesters that occur during ischaemia and hypoxia (Severson, 1979; Katz & Messineo, 1982).

The apparent dissociation constant of palmitate binding by liver cytosolic protein of about 1 μM is identical to that observed with equilibrium-dialysis studies (Ketterer *et al.*, 1976), but is threefold lower than that reported for oleate on the basis of gel-filtration experiments (Mishkin *et al.*, 1972). With equilibrium-dialysis, the total fatty acid-binding capacity of liver cytosol was found to be only 0.1 pmol/μg protein (Ketterer *et al.*, 1976). Purified FABP from pig heart showed an apparent dissociation constant for palmitate of 0.85 μM, as measured by electron spin resonance spectroscopy (Fournier *et al.*, 1983a). This value is similar to that

found for rat heart cytosol (Table 10.1).

The simultaneous rise of the capacities of cytosolic fatty acid binding and of palmitate oxidation during the dark relative to the light period in both liver and heart corresponds to the concomitant changes of these parameters in various other conditions. The higher FABP concentration in cytosol from livers of female compared to male rats (Ockner *et al.*, 1980, 1982) correlated with a higher oleate oxidation by hepatocytes obtained from the female rats (Ockner *et al.*, 1980). The 2-fold rise of the hepatic FABP concentration observed after clofibrate administration (Fleischner *et al.*, 1975; Renaud *et al.*, 1978; Kawashima *et al.*, 1982) is also paralleled by a 2.5-fold rise of the palmitate oxidation capacity (chapter 6). Finally, during diabetes or starvation, both the total capacity of oleate binding to liver cytosolic proteins (Brandes & Arad, 1983) and the hepatic fatty acid oxidation (McGarry *et al.*, 1975; chapter 6) increase markedly. We found, however, no evidence for the presence of a fatty acid-binding component of $M_r$ 400,000 during the dark period, as observed during diabetes or starvation (Brandes & Arad, 1983).

The 2-fold diurnal variations of the cytosolic FABP content in liver and heart (Table 10.1) are smaller than the 10-fold diurnal fluctuation found for $SCP_1$ in liver (McGuire *et al.*, 1983). However, the maximum levels are of comparable magnitude, amounting to 70-80 (FABP) and about 100 ($SCP_1$; McGuire *et al.*, 1983) µg/mg protein. Feeding rats a cholestyramine containing diet results in an increase of both the rate of triacylglycerol synthesis from [U-$^{14}$C]palmitate and from [1,3-$^{3}$H]glycerol in hepatocytes (Kempen *et al.*, 1983b), and of the FABP content of liver cytosol, when the rats are sacrificed during the light period (Table 10.1). This suggests that the binding protein is involved in modulation of fatty acid incorporation into triacylglycerols.

Recently. Morin *et al.* (1982) showed that feeding of cholestyramine to adult rats increased the activities of hepatic $SCP_1$ and $SCP_2$. Furthermore, Dempsey *et al.* (1980) found an increase of similar size in $SCP_1$ level induced by partial ileal bypass, a manoeuvre also resulting in breaking the bile salt enterohepatic circulation. The virtual disappearance of the diurnal rhythm of the amount of FABP upon cholestyramine administration may relate to the finding that the amplitude of the diurnal rhythm of hepatic cholesterogenesis was markedly decreased after cholestyramine feeding (Weis

& Dietschy, 1975). All these results are compatible with the idea that FABP and $SCP_1$ are closely related or identical proteins, as suggested already by others (Billheimer & Gaylor, 1980; Dempsey *et al.*, 1981; Ockner *et al.*, 1982).

In heart, the diurnal fluctuation of the cytosolic content of FABP is accompanied by a change in that of endogenous fatty acids. In liver, we did not observe such a combination, as the cytosolic fatty acid content did not differ between the dark and the light period. This may be due to the binding by FABP of other lipidic compounds, such as mono-, di- and triacylglycerols and cholesterol and its precursors (Burnett *et al.*, 1979; Ockner *et al.*, 1982; Rüstow *et al.*, 1982). Evidence for the binding of compounds other than fatty acids is also obtained from a comparison of the palmitate binding by delipidated and non-delipidated cytosolic proteins (Fig. 10.2). If fatty acids were the only endogenous ligands present on FABP, their amount can be estimated from the observed degree of label dilution to be about 2 nmol/mg protein in liver cytosol, which amount is higher than the measured value (1.4 - 1.7 nmol/mg protein).

Diurnal changes of palmitate oxidation capacity and of citrate synthase activity, as we observed in liver and to a lesser extent also in heart, were to our knowledge not earlier reported. A diurnal variation of the triacylglycerol and free fatty acid content of total rat heart has been found by Garthwaite *et al.* (1979), but with maximal levels at the very beginning of the dark period and no differences between the mid-dark and mid-light levels. Hepatic fatty acid synthesis is known to show a diurnal rhythm with a peak activity during the dark period (Kimura *et al.*, 1970). The established correlation between the fatty acid oxidation capacity and the FABP content, and the demonstration that FABP-bound fatty acids can be transferred to the mitochondrial β-oxidative system (Fournier *et al.*, 1978) indicate that FABP participates in the regulation of fatty acid metabolism and especially of oxidation. The high fatty acid-binding capacity in both liver and heart suggests that further studies on the role of FABP in these metabolically different tissues are of much interest.

## 10.5. SUMMARY

Delipidated proteins from albumin-free liver and heart cytosol obtained from rats sacrificed at the mid-dark or the mid-light phase of the light cycle were assayed for their palmitate binding capacity. In both tissues a marked variation of this binding capacity was observed from about 3-4 nmol/mg protein in the mid-light phase of the cycle to about 7-8 nmol/mg protein in the mid-dark phase.

Sephadex G-75 chromatography of the cytosolic proteins revealed that the palmitate binding could in all cases almost entirely be attributed to proteins of $M_r$ 12,000-14,000, suggesting that the observed diurnal variations are related to differences in the content of fatty acid-binding protein (FABP). In both rat liver and heart FABP represents about 4 (mid-light) to 8% (mid-dark) of the total soluble proteins.

Cholestyramine feeding increased the FABP content of liver cytosol from rats sacrificed at the mid-light phase, but not in those sacrificed at the mid-dark phase, in such a way that the diurnal variation of the FABP content virtually disappeared.

The palmitate oxidation capacity and citrate synthase activity also exhibited a concomitant diurnal periodicity in rat liver and, to a lesser extent, in rat heart.

The results provide additional evidence for an important role of FABP in cellular fatty acid metabolism in both liver and heart and for the similarity of FABP with sterol carrier protein.

# CHAPTER 11

# PURIFICATION AND CHARACTERIZATION OF FATTY ACID-BINDING PROTEIN FROM RAT HEART AND LIVER

## 11.1. INTRODUCTION

Fatty acid-binding protein (FABP) has now been purified and characterized from a number of tissues of several animal species and man, although most attention was paid to the protein from rat liver (see section 1.5). All proteins studied so far were found to have a similar molecular weight (11,000-15,000), but showed isoelectric points varying from 4.8 to 7.6 (Table 1.3). In addition, several groups reported that purified rat liver FABP preparations comprise three proteins with an identical $M_r$, but with different p*I* values (Ketterer *et al.*, 1976; Billheimer & Gaylor, 1980; Trulzsch & Arias, 1981). However, evidence is accumulating that rat liver FABP is a virtually neutral protein (p*I* 7.0 - 7.5) and that the acidic forms represent denatured protein or arise from the binding of various kinds of ligands (Dempsey *et al.*, 1981; Trulzsch & Arias, 1981). Binding of long-chain fatty acids to bovine liver FABP (Haunerland & Spener, 1980) and human serum albumin (Basu *et al.*, 1978) was reported to be accompanied by a low p*I* value. Therefore, delipidation of the proteins appears to be an important prerequisite for the proper characterization of FABP.

Immunochemical cross-reactivity of FABPs from different tissues has been observed. An antiserum raised agianst purified rat liver FABP was reactive with the cytosol of intestine, adipose tissue, heart, skeletal muscle, kidney and testis (Ockner & Manning, 1974; Ketterer *et al.*, 1976; Rüstow *et al.*, 1982), and that against rat jejunal FABP was reactive with the $M_r$ 12,000 fraction of liver, adipose tissue and heart cytosol (Ockner & Manning, 1974). However, the FABPs from human liver and adipose tissue are immunochemically different from those in the corresponding rat tissues (Rüstow *et al.*, 1982; Ul-Haq *et al.*, 1983). The possibility that other closely related but immunochemically distinct FABPs are also present in the va-

rious tissues is, however, not excluded.

In the previous chapter we showed that rat heart cytosol exhibits a fatty acid-binding capacity of similar magnitude to that of liver. FABP from rat and pig heart has only been studied by Fournier and co-workers (1978, 1983a,b). In order to compare the molecular properties of rat heart and liver FABP we purified and partially characterized FABP from both tissues. The results indicate that heart and liver FABP have a similar $M_r$ as deduced from SDS-polyacrylamide gel electrophoresis, but show different p*I* values.

## 11.2. MATERIALS AND METHODS

### *Purification of FABP*

Male albino Wistar rats, weighing 180-220 g, were used. The rats were fed ad libitum and sacrificed during the light phase of the diurnal cycle (chapter 10). Rat liver and heart cytosol were prepared as described in section 2.2. No attempt was made to remove residual albumin, which is present in the heart cytosol, but not in the liver cytosol (section 10.2). The cytosolic proteins were delipidated by chromatography on Lipidex at 37°C, as described in section 10.2, and stored at -20°C until use. All further steps in the purification of FABP were performed at 4°C. The delipidated cytosol (40-100 mg protein) was applied to a column of Sephadex G-75 (1.6 × 85 cm), equilibrated with 30 mM Tris-HCl (pH 8.0). The flow rate was 20 ml/h and 3.7-ml fractions were collected. Fractions containing FABP activity (no. 31-36; Fig. 10.5, page 146) were combined, concentrated by ultrafiltration using a Diaflo UM-2 or PM-10 membrane (Amicon Corporation, Lexington, MA, U.S.A.), and applied to a DEAE-Sephacel column (1.8 × 12 cm), equilibrated with 30 mM Tris-HCl (pH 8.0). The flow rate was 35 ml/h and the fraction volume 3.8 ml. The column was washed with the same buffer until the first protein peak was completely eluted (Fig. 11.1). Retained protein was then eluted with a linear gradient (2 × 70 ml) of 0 - 0.3 M KCl in 30 mM Tris-HCl (pH 8.0).

Protein was located in chromatographic fractions by measuring the $A_{280}$ and quantitated as described in section 2.3. Assay of palmitate binding was performed as described in section 8.2, using 50-200-µl samples and 1 µM [1-$^{14}$C]palmitate.

*Polyacrylamide slab gel electrophoresis*

FABP-containing fractions at various stages of purification were subjected to polyacrylamide slab gel electrophoresis in SDS (15% (w/v) acrylamide, of which 3% (w/v) bisacrylamide, and 0.1% (w/v) sodium dodecyl sulphate at pH 8.8) according to the method of Laemmli (1970). The Pharmacia Gel Electrophoresis Apparatus GE-2/4 was used (Pharmacia Fine Chemicals, Uppsala, Sweden). Each slot was loaded with about 5-25 µg of protein. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 (Merck, Darmstadt, F.R.G.) in methanol/acetic acid/water (50:7:43, by vol) and destained with methanol/acetic acid/water (5:7:88, by vol).

*Isoelectric focusing*

Isoelectric focusing was carried out in gel rods using the same apparatus as for gel electrophoresis and Parmalyte ampholytes (pH 3 to 10; Pharmacia Fine Chemicals, Uppsala, Sweden). The anode and cathode solutions were 10 mM phosphoric acid and 20 mM NaOH, respectively. Protein samples (5-10 µg per gel rod) were subjected to focusing at 400 V. The gel rods were stained with 0.1% Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water (25:8:67, by vol) and destained with the same solution without the dye.

## 11.3. RESULTS

*Purification of FABP from rat heart and liver*

Fatty acid-binding protein was purified from the delipidated cytosol of rat heart and liver by gel-filtration and ion-exchange chromatography. Following fractionation on Sephadex G-75, fatty acid-binding activity was with both preparations mainly present in fractions corresponding to a molecular weight of 10,000-15,000, but some activity was also found in the region of $M_r$ 1500-2000 (elution profiles are shown in Fig. 10.5, pages 146 and 147). The latter fractions, that contain a fatty acid-binding peptide (Suzue & Marcel, 1975; Rustow *et al.*, 1978, 1979), were not further studied. With rat heart cytosol, fatty acid-binding activity was usually also present in the high-$M_r$ region ( $> M_r$ 50,000), due to the presence of residual albumin (cf. chapter 10). This activity peak was, however, always completely separated from the FABP-containing fractions ($M_r$ 10,000-20,000 proteins).

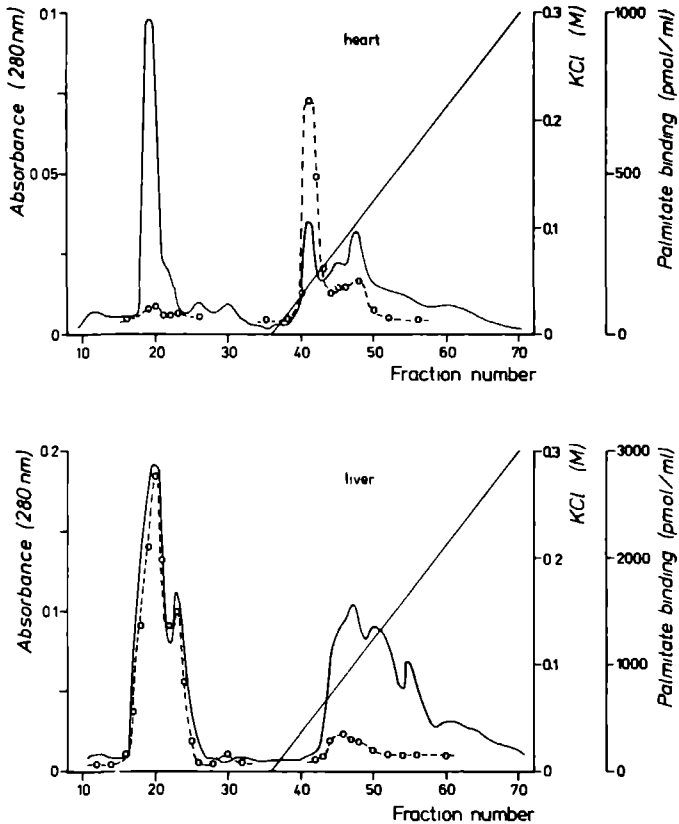



Fig. 11.1. Purification of FABP from rat heart and liver on DEAE-Sephacel. The combined, FABP-containing fractions of the Sephadex G-75 column were chromatographed on DEAE-Sephacel with 30 mM Tris-HCl, pH 8.0 (up to fraction 36) and subsequently with a linear gradient of 0 - 0.3 M KCl in the same buffer, as described in section 11.2.
Fractions were assayed for protein by measuring $A_{280}$ (—) and for palmitate binding (o). In order to trace myoglobin, the protein fractions from heart were also measured at 434 nm. Absorbance at this wavelength was only found with fractions 18-23.
The results are shown of representative experiments, in which 1.6 mg (heart) and 2.9 mg (liver) of protein were applied to the column.

The combined FABP-containing fractions were further purified by anion-exchange chromatography on DEAE-Sephacel at pH 8.0. As shown in Fig. 11.1, liver FABP was not retained by the column in contrast to heart FABP. The latter protein could be eluted from the column by applying a linear gradient of KCl. These observations indicate that liver FABP is a basic protein that has a p*I* which is higher than that of heart FABP.

Table 11.1. gives a summary of the purification procedure. For liver

*Table 11.1. Purification of fatty acid-binding protein from rat heart and liver.*

| | Protein (mg) | Specific fatty acid-binding activity [a] (pmol/μg protein) | Recovery of binding activity (%) | Purification (-fold) |
|---|---|---|---|---|
| HEART | | | | |
| Delipidated 105,000 × *g* supernatant | 39.7 | 3.6 [c] | 100 | 1 |
| Sephadex G-75 [b] | 11.4 | 4.3 | 35 | 1.2 |
| DEAE-Sephacel [b] | 2.0 | 18.4 | 26 | 5.2 |
| LIVER | | | | |
| Delipidated 105,000 × *g* supernatant | 129 | 2.3 | 100 | 1 |
| Sephadex G-75 [b] | 8.4 | 19.3 | 55 | 8.4 |
| DEAE-Sephacel [b] | 2.2 | 41.7 | 31 | 18.1 |

Heart and liver cytosol were obtained from rats sacrificed during the light phase of the diurnal cycle. The results given are representative for four independent experiments with each tissue.

[a] Determined with 1 μM [1-$^{14}$C]palmitate.

[b] The concentrating of the samples prior to chromatography gave a loss of protein of 20-25%.

[c] Fatty acid binding is also due to the presence of residual albumin in this preparation (see text).

FABP a final purification factor of 18 was obtained and the overall recovery was 31% with respect to the delipidated cytosolic proteins. This purification factor is in accordance with the hepatic content of FABP (during the light phase of the light cycle) of about 4-5% of the cytosolic proteins (Ockner *et al.*, 1982; sections 8.4 and 10.3). For heart FABP the purification factor is much lower, although the cytosolic content of FABP in this tissue appears comparable to that of liver (section 10.3). This lower value can partly be explained by underestimation of the purification factor, due to the presence of albumin in the cytosol preparation. As the palmitate binding activity of dealbuminized heart cytosol is 2.02 ± 0.30 pmol/μg protein (mean ± S.D. of 3 determinations; taken from chapter 10), the corrected purification factor would amount to about (18/2 =) 9. Hence, contaminating proteins are still present in the FABP preparation, or some loss of functional activity has occurred during the purification procedure.

The FABP-containing fractions at various stages of purification were examined by SDS polyacrylamide gel electrophoresis at pH 8.8. The fractions obtained after Sephadex G-75 chromatography of both the heart and liver cytosol showed only a few protein bands (Fig. 11.2, slot 3 and 7). After further purification on DEAE-Sephacel, heart FABP showed a single band on the gel, corresponding to $M_r$ 12,000 (Fig. 11.2. slot 5). The latter value is in agreement with observations by Fournier *et al.* (1978). The protein fraction not retained by the DEAE-Sephacel column at pH 8.0 mainly comprises myoglobin, as judged by gel electrophoresis (Fig. 11.2, slot 4) but also by its specific absorption at 434 nm (Fig. 11.1). The chromatographic behaviour corresponds to the reported p*I* values (7.5 - 8.5) for myoglobin from other animal species (Righetti & Caravaggio, 1976; Righetti *et al.*, 1981). Gel electrophoresis of the purified liver FABP, obtained after DEAE-Sephacel chromatography, revealed a major protein band corresponding to $M_r$ 12,000, which represents FABP (Ockner *et al.*, 1982; section 1.5), and some minor bands (Fig. 11.2, slot 8).

When purified heart and liver FABP were subjected to isoelectric focusing, single bands were observed at approximately pH 7.0 and 8.0, respectively (results not shown). These values are consistent with the behaviour of the proteins on DEAE-Sephacel chromatography.

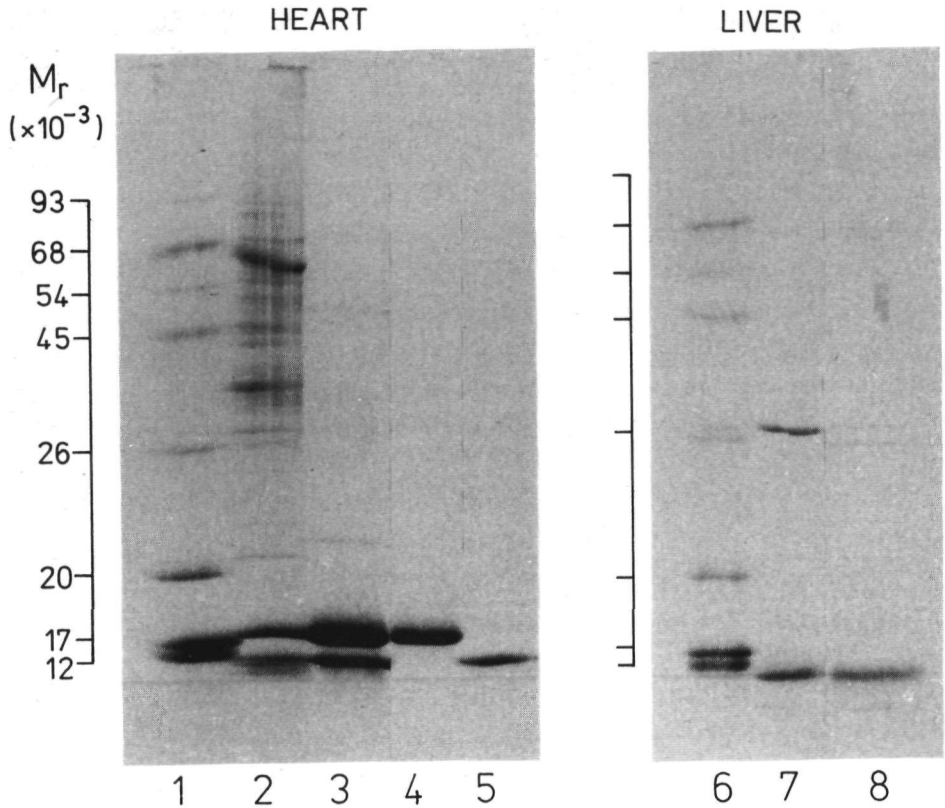



Fig. 11.2. Polyacrylamide slab gel electrophoresis in SDS at pH 8.8 of FABP-containing fractions from rat heart (slot 2-5) and liver (slot 7 and 8) at various stages of purification.
The numbers correspond to:
1, calibration proteins (with $M_r$): phosphorylase a (93,000), bovine serum albumin (68,000), leucine aminopeptidase (54,000), ovalbumin (45,000), α-chymotrypsinogen A (26,000), $A_2$ chain of calf α-crystallin (20,000), myoglobin (17,000) and cytochrome *c* (12,400).
2, delipidated heart cytosol (25 µg protein).
3, combined FABP-containing fractions after Sephadex G-75 chromatography (25 µg protein).
4, fraction 20 from DEAE-Sephacel column (10 µg protein).
5, fraction 41 from DEAE-Sephacel column (7 µg protein).
6, calibration proteins (see 1).
7, combined FABP-containing fractions after Sephadex G-75 chromatography (5 µg protein).
8, fraction 21 from DEAE-Sephacel column (5 µg protein).

*Fatty acid binding by the purified FABP preparations*

The binding of palmitate by FABP from heart as well as from liver was proportional to the amount of protein up to the binding of about 40-50% of the available fatty acid (Fig. 11.3), as was also observed with albumin (Fig. 8.2) and delipidated cytosolic proteins of both tissues (Fig. 10.2). In contrast to cytosolic protein preparations (chapter 10), palmitate binding activity of purified FABP decreased upon storage of the protein samples at -20°C, either lyophilized or in buffer solution (10 mM K-phosphate, pH 7.4) with or without 10% (v/v) glycerol. Loss of functional activity occurred sooner with heart FABP than with liver FABP.

When palmitate binding to FABP was studied as a function of the total palmitate concentration and the binding isotherms analyzed by means of a Scatchard plot (cf. chapter 10), a single class of saturable binding sites was observed (data not shown). With freshly purified heart FABP the apparent $K_d$ for palmitate was calculated to be 0.47 µM and the binding capacity 53 pmol/µg FABP (one preparation, tested in triplicate). For liver FABP the

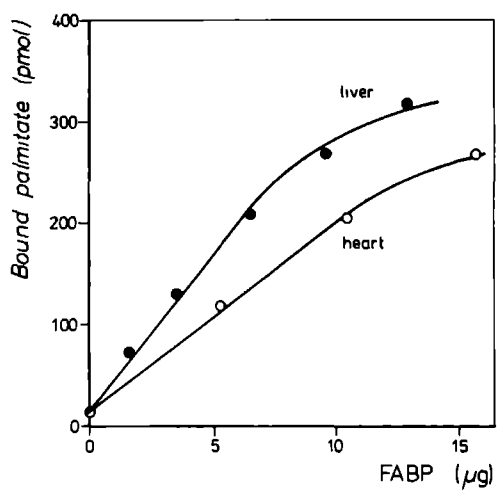



Fig. 11.3. Effect of the amount of purified FABP from rat liver (●) and heart (○) on the binding of palmitate. Measurements were performed with 1 µM [1-$^{14}$C]palmitate according to the procedure described in chapter 8. The results are shown of one representative experiment out of three.

apparent $K_d$ was 0.65 ± 0.06 μM and the binding capacity 79 ± 6 pmol/μg FABP (means ± S.D. for 3 fresh preparations). Since both FABPs have a $M_r$ of approximately 12,000 (Fig. 11.2), the binding capacity is about 0.6 and 0.95 ± 0.07 mol/mol FABP (heart and liver, respectively). The value for liver FABP indicates the presence of one fatty acid-binding site per protein molecule, as was earlier suggested (section 8.4). The binding capacity found for heart FABP will have been underestimated, due to loss of binding activity during purification.

## 11.4. DISCUSSION

The fatty acid-binding proteins isolated from heart and liver were found to have virtually identical molecular weights, as determined by SDS-polyacrylamide gel electrophoresis ($M_r$ about 12,000), but behaved distinctly on anion-exchange chromatography at pH 8.0. FABP from liver appeared to be a basic protein with a p*I* of approximately 8.0, which is higher than that of heart FABP (p*I* approximately 7.0). Our p*I* value for liver FABP is higher than the values reported by most other laboratories (Ketterer *et al.*, 1976; Matsushita *et al.*, 1977; Trulzsch & Arias, 1981; Ockner *et al.*, 1982; cf. Table 1.3, page 25). This difference is most likely caused by the absence of endogenous fatty acids in our preparations, since defatted preparations of bovine liver FABP (Haunerland & Spener, 1980) and of human serum albumin (Basu *et al.*, 1978) show a higher p*I* value than non-defatted samples. The acidic forms of FABP may also arise from the binding of endogenous ligands (Trulzsch & Arias, 1981; Ockner *et al.*, 1982). FABP from delipidated rat heart cytosol was, however, earlier reported to have a p*I* of 5.0 (Fournier *et al.*, 1978), but the delipidation procedure (treatment with 20% (v/v) butanol) used by these investigators is denaturing for FABP (Trulzsch & Arias, 1981).

One of the difficulties with the purification of heart FABP is its separation from myoglobin, which is present in relatively large quantities in rat heart (about 0.2 - 0.3 μmol/g wet weight tissue; Reynafarje, 1963; Schuder *et al.*, 1979), and also has a low molecular weight ($M_r$ 16,400). Because of the high p*I* value of myoglobin, complete separation of the two proteins could be achieved on anion-exchange chromatography at pH 8.0, as was also observed by Fournier *et al.* (1978). The purity of heart FABP was demonstrated on SDS-polyacrylamide gels, but did not appear from the palmi-

tate binding capacity (assuming one fatty acid-binding site per protein molecule), nor from the observed purification factor, when based on a cytosolic FABP content of about 4% (chapter 10). Therefore, purification of the protein was accompanied by loss of functional activity .

For both purified heart and liver FABP the apparent $K_d$ for palmitate is lower than that observed with the corresponding cytosolic protein samples (about 0.8 and 1.0 μM for heart and liver cytosolic protein, respectively; chapter 10). This may relate to a hampered fatty acid binding by the latter preparations, due to the presence of large amounts of contaminating proteins. The observed apparent dissociation constants are slightly lower than found for purified rat liver FABP by equilibrium-dialysis studies ($K_d$ = 1 μM; Ketterer *et al.*, 1976) and for purified pig heart FABP by electron spin resonance spectroscopy ($K_d$ = 0.85 μM; Fournier *et al.*, 1983a). The affinity of FABP for palmitate appears of the same order of magnitude as that reported for the high-affinity binding sites of albumin (Spector, 1975).

It is clear that further studies are necessary to define the conditions under which FABP does not loose its functional property during and after purification, and to more completely characterize the proteins from rat heart and liver. An additional purification step may also be considered to remove the few contaminating proteins that were found to be still present in the liver FABP preparation (Fig. 10.2). Amino acid analyses and cross-reactivity of antibodies raised against the liver and heart FABP may reveal similarities and differences between the two proteins. Finally, purified FABP preparations can be used as a tool for the study of its role in cellular fatty acid metabolism.

## 11.5. SUMMARY

Fatty acid-binding protein (FABP) was purified from the delipidated cytosol of rat heart and liver by gel-filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephacel at pH 8.0.

The homogeneity of heart FABP was demonstrated by a single band on SDS-polyacrylamide gels. In the liver FABP preparation some contaminating proteins were still present.

The heart and liver FABP have almost identical molecular weights ($M_r$ approximately 12,000; found with SDS-polyacrylamide gel electrophoresis), but the liver FABP is more basic (p*I* approximately 8.0) than the heart FABP (p*I* approximately 7.0).

Functional activity of FABP appeared to decrease during and after purification from liver and, especially, from heart.

# CHAPTER 12

# SURVEY AND SUMMARY

## 12.1. INTRODUCTION

Fatty acids represent the main fuel for energy production in both skeletal muscle and heart, especially during rest and prolonged exercise, and after fasting (section 1.3). Increasing our understanding of the pathway of fatty acid oxidation and its regulation will provide a better insight in the functioning of normal and pathological muscle. The biochemical investigations described in this thesis deal with two aspects of fatty acid oxidation in muscle: a comparison of the use of cell-free and cellular systems for oxidation measurements (chapters 3-7), and studies on the assay and the role of the fatty acid-binding protein in fatty acid metabolism (chapters 8-11).

## 12.2. FATTY ACID OXIDATION IN SKELETAL AND CARDIAC MUSCLE

With isolated mitochondria fatty acid oxidation rates can accurately be measured by polarographic determination of the oxygen consumption. For whole homogenates or cellular systems, however, this method appears unsuitable because of the interference of endogenous substrates and of the oxygen binding protein myoglobin. Therefore, with the latter systems fatty acid oxidation rates are usually determined radiochemically from the production of $^{14}CO_2$ from [$^{14}$C]fatty acids. In earlier studies from this laboratory (Van Hinsbergh *et al.*, 1978a,b, 1979, 1980), in which the polarographic and radiochemical assay were compared for muscle mitochondria, it was established that $^{14}CO_2$ production can not be taken as an accurate measure for fatty acid oxidation rates. Calculation of the oxidation rate from the sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products, formed during oxidation of [$^{14}$C]fatty acids, was found to agree better with the polarogra-

phically determined rate and to be more sensitive. The latter radiochemical assay procedure appeared also suitable for the measurement of fatty acid oxidation rates in muscle whole homogenates and cellular systems (chapters 3 and 7). The contribution of the $^{14}CO_2$ production to the total oxidation rate was generally higher with the cellular than with the cell-free systems (Table 12.1).

*Table 12.1. Proportional contribution of $^{14}CO_2$ production to the [1-$^{14}$C]-palmitate oxidation rate by rat skeletal and cardiac muscle.*

| System | Diaphragm | Heart |
|---|---|---|
| Mitochondria | 10 ± 2 | 16 ± 2 |
| Homogenate | 12 ± 5 | 18 ± 3 |
| Slices / intact muscle | 53 ± 3 | 66 ± 8 |
| Myocytes | - | 68 ± 7 |

Values are given in % of the total sum of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products and are means ± S.D. of 5-25 experiments.

Although it is clear from the present and previous studies that fatty acid oxidation rates can not accurately be determined only from the production of $^{14}CO_2$ from [$^{14}$C]fatty acids, this method is still being used by various investigators (Paul & Adibi, 1979; Sumbilla *et al.*, 1981; Okano & Shimojo, 1982; Barakat *et al.*, 1982, 1983; Shumate *et al.*, 1982; Carroll *et al.*, 1983; Menon & Dhopeshwarkar, 1983). Comparison of $^{14}CO_2$ production rates from [$^{14}$C]fatty acids may in some cases (e.g. perfused heart preparation) permit valid conclusions, but in many cases results do not appear meaningful.

Feeding of clofibrate, an effective hypolipidemic and hypocholesterolemic drug, to rats was found to predominantly affect oxidative metabolism in rat liver and hardly in skeletal muscle (chapter 6). These observations disprove those of Paul & Adibi (1979, 1980), who based their conclusions on $^{14}CO_2$ production rates, and indicate that myopathic phenomena observed after chronic clofibrate administration are not related to changes in the capacity of oxidative metabolism of muscle.

The developed assay for fatty acid oxidation was applied to study the postnatal development of palmitate oxidation in both intact preparations of rat skeletal muscle and homogenates of skeletal and cardiac muscle (chapter 4). Palmitate oxidation by hemidiaphragm and intact m.flexor digitorum brevis gradually decreases with development to adult values which are about 35% of those at birth. The oxidation capacities of diaphragm, m.quadriceps and, particularly, of heart increase steadily during development, together with the activities of the mitochondrial marker enzymes cytochrome *c* oxidase and citrate synthase. In heart the increase of the oxidative capacity results from an increase of both mitochondrial content and mitochondrial activity, but in diaphragm and m.quadriceps only from a change of the mitochondrial activity.

With cell-free preparations it was previously observed (Van Hinsbergh *et al.*, 1978a,b, 1979, 1980) that the oxidation rate of [$^{14}$C]palmitate depends upon the position of the $^{14}$C-label in the palmitate molecule. [1-$^{14}$C]-palmitate delivers a larger amount of $^{14}CO_2$ and $^{14}$C-labeled acid-soluble products than [U-$^{14}$C]- or [16-$^{14}$C]palmitate, which indicates incomplete oxidation of the palmitate molecule and hence accumulation of β-oxidation intermediates. Analysis of the oxidation products of incubations with [16-$^{14}$C]palmitate by homogenates and mitochondria of rat m.quadriceps and liver revealed that mainly dodecanoic and tetradecanoic acid and their esters accumulate (chapter 5). Their amounts recovered can account for the differences in oxidation rates with [1-$^{14}$C]- and [16-$^{14}$C]palmitate. With intact muscular preparations, however, the oxidation rate was not dependent on the position of the $^{14}$C-label in the palmitate molecule. From experiments in which we studied the influence of various parameters on the oxidation of [1-$^{14}$C]- and [16-$^{14}$C]palmitate, and the demonstration of the occurrence of peroxisomal fatty acid oxidation in muscle, it appears that the incomplete palmitate oxidation in cell-free systems is mainly caused by an inadequate mitochondrial oxidation of peroxisomal oxidation products.

With rat hemidiaphragm the palmitate oxidation rate increases considerably with the external palmitate concentration and the palmitate:albumin molar ratio throughout the range of physiologically relevant concentrations (chapter 3). Palmitate oxidation rates were also concentration dependent in human and rat heart slices, but not with calcium-tolerant rat cardiac myocytes (chapter 7). When measured at 0.4 mM albumin, the apparent $K_m$ for palmitate oxidation was with hemidiaphragm from 18-h starved rats 2.8 mM

*Table 12.2. Kinetic parameters of the concentration dependence of palmitate oxidation in rat muscle.*

| System | Apparent $K_m$ (mM) | $V_{max}$ (nmol/min per g) |
|---|---|---|
| Heart homogenate | 0.27 | 891 |
| Heart slices | 1.45 | 17 |
| M.quadriceps homogenate | 0.36 | 249 |
| Diaphragm homogenate | 0.37 | 701 |
| Hemidiaphragm | 2.76 | 32 |
| Hemidiaphragm (3 days starved) | 0.98 | 25 |

Parameters were calculated from Lineweaver-Burk plots of oxidation rates measured at 0.4 mM albumin (with heart slices at 0.3 mM albumin).

and decreased after prolonged starvation of the animals (Table 12.2). The $K_m$ values were generally lower in homogenates of skeletal and cardiac muscle than in intact cellular systems. These differences can largely be explained by variations in the degree of label dilution of the added [$^{14}$C]-palmitate with free fatty acids derived from endogenous lipid stores (chapters 3 and 7). The maximal rate of palmitate oxidation by hemidiaphragm depends hardly on the albumin concentration in the medium, does not change after prolonged starvation, and is about equal to the rate of endogenous free fatty acid consumption, determined from the release of glycerol (25-30 nmol/min per g in all cases). Palmitate oxidation by intact muscles appears to be hampered by a slow diffusion of substrate in the muscle, since the oxidation rates, when based on citrate synthase activities, are much higher with isolated rat cardiac myocytes than in heart slices. The oxidation capacities of various rat skeletal muscles and of human and rat heart, determined with whole homogenates, are severalfold higher than the oxidation rates with intact preparations (Table 12.2).

## 12.2. FATTY ACID-BINDING PROTEIN OF RAT HEART AND LIVER

Fatty acid-binding protein (FABP) has been identified in many tissues that utilize fatty acids (section 1.5). This protein of $M_r$ 12,000 is assumed to participate in the intracellular transport and/or storage of fatty acids, much like albumin does extracellularly. FABP may thus function in mitochondrial and peroxisomal fatty acid oxidation, triacylglycerol synthesis and degradation and hepatic cholesterol esterification (Fig. 12.1), but also inhibit the detrimental effects exerted by fatty acids and their derivatives (Severson, 1979). In heart, high intracellular levels of fatty acids and fatty acyl-CoA esters, occurring during hypoxia and ischaemia, have been demonstrated to be involved in the disruption and distorion of various cellular membrane processes related to energy metabolism and $Ca^{2+}$ homeostasis, such as the inhibition of the mitochondrial adenine nucleotide translocase.

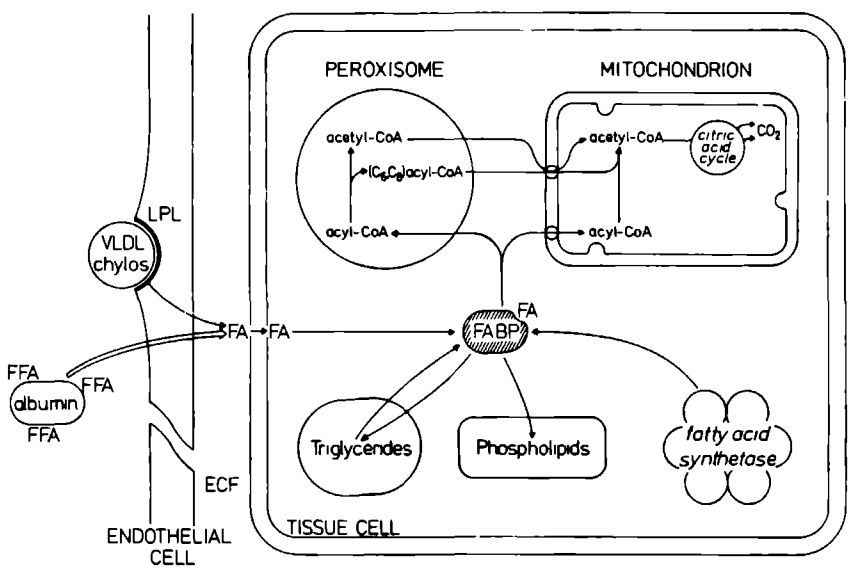



Fig. 12.1. Putative role of fatty acid-binding protein (FABP) in the cellular metabolism of long-chain fatty acids.
VLDL, very low density lipoproteins; chylos, chylomicrons; LPL, lipoprotein lipase; (F)FA, (free) fatty acid; ECF, extracellular fluid.

A radiochemical procedure was developed for the sensitive assay of fatty acid binding by proteins (chapter 8). In this assay protein-bound and unbound fatty acids are separated by binding the latter to a hydrophobic column-packing material, Lipidex 1000, at 0°C. This low temperature appeared essential since at a higher temperature protein-bound fatty acids are also removed from the solution, in accordance with the protein-lipid interaction kinetics. This latter property could, however, favourably be applied for the effective delipidation of protein samples (chapter 8) and the defatting of commercial preparations of serum albumin (chapter 9). Lipidex is particularly suitable since it does not bind protein, in contrast to charcoal.

With the radiochemical assay we obtained results on palmitate binding to serum albumin similar to those reported on the basis of equilibrium-dialysis (chapter 8). Palmitate binding by cytosolic proteins from rat heart and liver was proportional to the amount of protein, only when delipidated protein samples were used (chapter 10). Furthermore, after delipidation the palmitate binding per µg protein was higher than with non-delipidated samples, when measured at a low fatty acid concentration. The observations show that delipidation is important for the accurate determination of fatty acid-binding activities and that Lipidex is well suitable for this treatment.

The palmitate binding capacity of delipidated proteins from albumin-free rat heart and liver cytosol was about 2-fold higher when the animals were sacrificed at the mid-dark phase compared to the mid-light phase of the light cycle (chapter 10). By Sephadex G-75 chromatography of the cytosolic proteins it was established that this binding can almost entirely be attributed to the presence of FABP. Evidence was also obtained that FABP possesses only one fatty acid-binding site per protein molecule. From the maximal palmitate binding by the cytosolic proteins we calculated that both rat heart and liver contain about 35-40 and 70-80 µg FABP/mg cytosolic protein at the mid-light and mid-dark phase, respectively. The affinity of the protein for palmitate is similar to that of albumin ($K_d$ = 0.5 - 1 µM). The palmitate oxidation capacity and citrate synthase activity also exhibited a concomitant diurnal periodicity in rat liver and, to a lesser extent, in rat heart.

FABP was purified to homogeneity from both rat heart and liver by gel-filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephacel

at pH 8.0 (chapter 11). The FABPs from both tissues have an almost identical molecular weight ($M_r$ 12,000), but the liver FABP appears to be more basic (p*I* approximately 8.0) than the heart FABP (p*I* approximately 7.0).

Previous estimations of the FABP content of rat heart cytosol have been too low, due to the use of a less suitable binding assay or antibodies raised against liver FABP. The latter do likely not show complete cross-reactivity with heart FABP, since both proteins have different molecular properties. Because of its high cytosolic concentration and its remarkable diurnal variation we suggest that FABP may play an important role in cellular lipid metabolism by modulating the availability of fatty acids and their derived acyl esters. In this way the protein may indirectly be involved in many processes of cellular metabolism.

A major part of this thesis is concerned with methodological aspects of the measurement of fatty acid oxidation in various muscle preparations and the detection and purification of FABP. On the basis of these studies, further investigations are feasible, in which the role of FABP in muscular fatty acid metabolism will be examined more directly.

# SAMENVATTING

## VETZUUROXIDATIE IN SKELET- EN HARTSPIER

Vetzuren leveren de belangrijkste bijdrage aan de energie voorziening in skelet- en hartspier van mens en dier, met name tijdens rust, bij langdurige inspanning en na vasten. Onze kennis omtrent het verloop en de regulatie van de vetzuuroxidatie in de spier is beperkt, met name bij de mens. Uitbreiding van deze kennis zal leiden tot een beter inzicht in het functioneren van de normale en pathologische spier en tevens mogelijkheden geven tot diagnostiek en eventueel therapie van metabole spierziekten. Het biochemisch onderzoek dat in dit proefschrift wordt beschreven betreft twee aspecten van de vetzuuroxidatie in de spier: (a) een vergelijking van het gebruik van celvrije en cellulaire systemen voor oxidatiemetingen (hoofdstukken 3-7), en (b) de bestudering van de bepaling en de rol van vetzuurbindende eiwitten in het vetzuurmetabolisme (hoofdstukken 8-11).

In hoofdstuk 1 wordt de morfologie van de skelet- en hartspier vergeleken en wordt een overzicht gegeven van de huidige kennis omtrent het gebruik van energie-leverende substraten door de dwarsgestreepte spier, en omtrent de fysisch-chemische eigenschappen en functionele aspecten van intracellulaire vetzuurbindende eiwitten. In hoofdstuk 2 worden de materialen en enkele methodes beschreven, die bij het verdere onderzoek veelvuldig werden gebruikt. Deze methodes omvatten de preparatie van de verschillende cellulaire en celvrije systemen, de bepaling van de activiteit van enkele enzymen en de meting van glycerol en vrije vetzuren.

Polarografische meting van het zuurstof verbruik is niet geschikt voor vetzuuroxidatie metingen aan homogenaten of cellulaire systemen, mede omdat deze meting gestoord wordt door de verbranding van endogene substraten en door de aanwezigheid van het zuurstofbindend eiwit myoglobine. Daarom wordt de vetzuuroxidatie hierin meestal radiochemisch bepaald uit de vorming van $^{14}CO_2$ uit [$^{14}$C]vetzuren. Uit eerdere studies van dit laboratorium, waarin de polarografische en radiochemische methodes werden vergele-

ken, bleek echter dat de $^{14}CO_2$ productie geen goede maat is voor de vetzuuroxidatiesnelheid van geïsoleerde mitochondriën. Wanneer de oxidatiesnelheid wordt berekend uit de som van $^{14}CO_2$ en $^{14}C$-gelabelde zuur-oplosbare producten, werd een betere overeenstemming gevonden met metingen van het zuurstof verbruik. Deze radiochemische methode bleek nu ook bruikbaar voor de bepaling van de vetzuuroxidatiesnelheid in homogenaten en cellulaire spiersystemen (hoofdstukken 3 en 7). De bijdrage van de $^{14}CO_2$ productie aan de totale vetzuuroxidatiesnelheid was met celvrije systemen minder dan 20%, maar met cellulaire systemen 30-70%.

Toediening van het bloed-lipide gehalte verlagende geneesmiddel clofibraat aan ratten bleek hoofdzakelijk het oxidatief metabolisme in de lever te beïnvloeden, en nauwelijks dat in de skeletspier (hoofdstuk 6). Deze bevindingen weerleggen die uit de literatuur, welke gebaseerd werden op bepaling van uitsluitend $^{14}CO_2$ productie.

De radiochemische methode is ook toegepast voor de bestudering van de postnatale ontwikkeling van de palmitaat oxidatie (hoofdstuk 4). De oxidatiecapaciteit van diafragma, m.quadriceps en vooral van hartspier nemen in de rat na de geboorte aanzienlijk toe. Deze toename gaat gepaard met een toename van de activiteiten van de mitochondriële enzymen cytochroom *c* oxidase en citraat synthase. De toename van de oxidatiecapaciteit is in hart het gevolg van een toename van zowel het mitochondrieel gehalte als de mitochondriële activiteit, maar in de twee skeletspieren te danken aan uitsluitend een toename van de mitochondriële activiteit.

In celvrije systemen is de hoeveelheid radioactiviteit, die gevonden wordt in $CO_2$ en zuur-oplosbare producten bij oxidatie van [$^{14}C$]palmitaat, afhankelijk van de positie van het label in het palmitaatmolecuul (hoofdstuk 5). Deze hoeveelheid is groter met [1-$^{14}C$]- dan met [16-$^{14}C$]palmitaat, hetgeen duidt op accumulatie van β-oxidatie intermediairen. Deze intermediairen bleken hoofdzakelijk dodecaan- en tetradecaanzuur en hun esters te zijn. Met intacte spierpreparaten trad geen accumulatie op. Uit studies, waarbij gekeken is naar de invloed van verschillende parameters op deze accumulatie en het feit dat peroxisomale vetzuuroxidatie kon worden aangetoond in de spier, is geconcludeerd dat de onvolledige palmitaatoxidatie in celvrije systemen voornamelijk wordt veroorzaakt door een onvoldoende mitochondriële oxidatie van peroxisomale oxidatieproducten.

De oxidatie capaciteiten gemeten aan homogenaten van humaan en rattehart en van verschillende skeletspieren van de rat zijn aanzienlijk hoger

dan de oxidatiesnelheden gemeten met coupes of intacte spieren (hoofdstukken 3 en 7). Gemeten bij een constante albumine concentratie is de schijnbare $K_m$ voor palmitaat oxidatie over het algemeen lager in homogenaten dan in cellulaire systemen, als gevolg van verschillen in labelverdunning van het toegevoegde palmitaat met vetzuren van endogene oorsprong. De palmitaat oxidatiesnelheid in hemidiafragma is sterk afhankelijk van de vetzuurconcentratie in het medium en van de vetzuur:albumine verhouding (hoofdstuk 3). Een dergelijke afhankelijkheid is ook waargenomen met coupes van humaan en rattehart, maar niet met geïsoleerde hartcellen van de rat (hoofdstuk 7). De vetzuuroxidatie gemeten met intacte spiersystemen wordt gehinderd door de langzame diffusie van het vetzuur in het spierweefsel. In geïsoleerde hartcellen bleek de palmitaatoxidatiesnelheid hoger dan in hartcoupes van de rat, wanneer de oxidatiesnelheden werden gerelateerd aan de citraatsynthase activiteit (hoofdstuk 7).

In hoofdstuk 8 wordt een geschikte radiochemische methode beschreven voor de bepaling van de vetzuurbinding aan eiwitten. Bij deze methode worden eiwitgebonden en vrije vetzuren gescheiden door binding van de laatste aan een hydrofoob kolommateriaal, Lipidex 1000, bij 0°C. Van de eigenschap dat bij een hogere temperatuur de eiwitgebonden vetzuren ook worden onttrokken kan gebruik gemaakt worden voor het effectief delipideren van eiwitfracties (hoofdstuk 8) en voor het ontvetten van commerciële albumine preparaten (hoofdstuk 9). De met deze methode bepaalde vetzuurbinding aan albumine gaf resultaten, die vergelijkbaar zijn met literatuurgegevens (hoofdstuk 8). De palmitaatbinding aan cytosolische eiwitten van hart en lever van de rat is alleen evenredig met de hoeveelheid eiwit, wanneer gedelipideerde eiwitmonsters worden gebruikt (hoofdstuk 10).

De vetzuurbindende capaciteit van de cytosolische eiwitten van hart en lever is twee keer zo hoog wanneer de ratten worden gedood in het midden van de donkerfase in vergelijking met de lichtfase (hoofdstuk 10). Deze vetzuurbinding kon grotendeels toegeschreven worden aan de aanwezigheid in het cytosol van het vetzuurbindend eiwit van $M_r$ 12,000. Aangezien dit eiwit slechts één bindingsplaats voor vetzuren heeft (hoofdstuk 11), kon worden berekend dat zowel in hart als in lever het vetzuurbindend eiwit in de lichtfase ongeveer 4 en in de donkerfase ongeveer 8% van de cytosolische eiwitten uitmaakt.

In hoofdstuk 11 is de zuivering van vetzuurbindende eiwitten uit rattehart en lever beschreven. De eiwitten uit beide weefsels hebben een

zelfde molecuulgewicht ($M_r$ 12,000), maar het lever eiwit is basischer (p*I* ongeveer 8.0) dan het eiwit uit de hartspier (p*I* ongeveer 7.0).

Het hoge vetzuurbindend eiwit gehalte van hart en lever cytosol en de opmerkelijke dagelijkse schommelingen daarin doen vermoeden dat dit type eiwit een belangrijke rol speelt in het lipide metabolisme van de cel door regulering van de beschikbaarheid van vetzuren en hun acyl esters.

# REFERENCES


Abu-Erreish, G.M. & Sanadi, D.R. (1978) *Mech. Ageing Dev.* 7, 425-432

Abumrad, N.A., Stearns, S.B.,Tepperman, H.M. & Tepperman, J. (1978) *J. Lipid Res.* 19, 423-432

Abumrad, N.A., Perkins, R.C., Park, J.H. & Park, C.R. (1981) *J. Biol. Chem.* 256, 9183-9191

Adler, R., Margyles, E., Motsan, R., Way, L. & Ockner, R.K. (1978) *Metabolism* 27, 607-613

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J.D. (1983) *Molecular Biology of the Cell*, Garland Publ. Inc., New York

Altschuld, R., Gibb, L., Ansel, A., Hohl, C., Kruger, F.A. & Brierley, G.P. (1980) *J. Mol. Cell. Cardiol.* 12, 1383-1395

Angelin, B., Einarsson, K., Hellström, K. & Leijd, B. (1978) *J. Lipid Res.* 19, 1017-1024

Appelkvist, E.L. & Dallner, G. (1980) *Biochim. Biophys. Acta* 617, 156-160

Aprille, J.R. (1976) *Pediat. Res.* 10, 784-787

Bahl, J.J., Navin, T.R. & Bressler, R. (1980) in *Carnitine Biosynthesis, Metabolism and Function* (Frenkel, R.A. & McGarry, J.D., Eds), pp. 91-113, Academic Press, New York

Bailey, E. & Lockwood, E.A. (1973) *Enzyme* 15, 239-253

Baldwin, K.M., Klinkerfuss, G.H., Terjung, R.L., Molé, P.A. & Holloszy, J. O. (1972) *Am. J. Physiol.* 222, 373-378

Baldwin, K.M., Cooke, D.A. & Cheadle, W.G. (1977) *J. Mol. Cell. Cardiol.* 9, 651-660

Baldwin, K.M., Hooker, K.M., Campbell, P.J. & Lewis, R.E. (1978) *Am J. Physiol.* 235, C 97-102

Ball, M.R., Gumaa, K.A. & McLean, P. (1979) *Biochem. Biophys. Res. Commun.* 87, 489-496

Barakat, H.A., Kasperek, G.J., Dohm, G.L., Tapscott, E.B. & Snider, R.D. (1982) *Biochem. J.* 208, 419-424

Barakat, H.A., Kasperek, G.J. & Dohm, G.L. (1983) *Biochem. Med.* 29, 298-306

Barbour, R.L. & Chan, S.H.P. (1979) *Biochem. Biophys. Res. Commun.* 89, 1168-1177

Basu, S.P., Rao, S.N. & Hartsuck, J.A. (1980) *Biochim. Biophys. Acta* 533, 66-73

Benassayag, C.,Valette, G., Delorme, J., Savu, L., Nunez, E.A. & Jayle, M. F. (1977) *Steroids* 30, 771-785

Berge, R.K. & Døssland, B. (1979) *Biochem. J.* 181, 119-125

Berger, M., Kemmer, F.W., Goodman, M.N., Zimmerman-Telchow, H. & Ruderman, N.B. (1978) in *Biochemical and Clinical Aspects of Ketone Body Metabolism* (Söling, H.D. & Seufert, C.D., Eds), pp. 193-203, Thieme Publ., Stuttgart

Bieber, L.L., Emaus, L., Valkner, K. & Farrell, S. (1982) *Fed. Proc.* 41, 2858-2862

Billheimer, J.T. & Gaylor, J.L. (1980) *J. Biol. Chem.* 255, 8128-8135

Björkhem, I. (1978) *J. Lipid Res.* 19, 585-590

Bloom, W. & Fawcett, D.W. (1975) *A Textbook of Histology*, pp. 288-332, 10th edition, Saunders, Philadelphia, PA

Bodel, P.T., Rubinstein, D., McGarry, E.E. & Beck, J.C. (1962) *Am. J. Physiol.* 203, 311-315

Borensztajn, J., Rone, M.S., Babirak, S.P., McGarr, J.A. & Oscai, L.B. (1975) *Am. J. Physiol.* 229, 394-397
Borum, P.R. (1978) *Biochem. J.* 176, 677-681
Brady, L.J. & Tandler, B. (1981) *Fed. Proc.* 40, 1624 (Abstr.)
Brandes, R. & Arad, R. (1983) *Biochim. Biophys. Acta* 750, 334-339
Brandes, R.,Ockner, R.K., Weisiger, R.A. & Lysenko, N. (1982) *Biochem. Biophys. Res. Commun.* 105, 821-827
Brass, E.P. & Hoppel, C.L. (1981) *Anal. Biochem.* 110, 77-81
Bremer, J. (1977) *Trends Biochem. Sci.* 2, 207-209
Bremer, J. & Wojtczak, A.B. (1972) *Biochim. Biophys. Acta* 280, 515-530
Bremer, J., Osmundsen, H., Christiansen, R.Z. & Borrebaek, B. (1981) *Meth. Enzymol.* 72, 506-519
Bridgman, J.F., Rosen, J.M. & Thorp, J.M. (1972) *Lancet* II, 506-509
Bronfman, M., Inestrosa, N.C. & Leighton, F. (1979) *Biochem. Biophys. Res. Commun.* 88, 1030-1036
Brooks, D.E. & McIntosh, J.E.A. (1975) *Biochem. J.* 148, 439-445
Burleigh, I.G. (1974) *Biol. Rev.* 49, 267-320
Burnett, D.A., Lysenko, N., Manning, J.A. & Ockner, R.K. (1979) *Gastroenterology* 77, 241-249
Bylund, A.C., Holm, J. & Scherstén, T. (1975) *Scand. J. Clin. Lab. Invest.* 35, 413-418
Capron, F., Coltoff-Schiller,B., Johnson, A.B., Fleischner, G.M. & Goldfischer, S. (1979) *J. Histochem. Cytochem.* 27, 961-966
Carlson, L.A., Ekelund, L.-G. & Fröberg, S.O. (1971) *Europ. J. Clin. Invest.* 1, 248-254
Carroll, J.E., Shumate, J.B., Villadiego, A., Choksi, R.M. & Morse, D.P. (1983) *Biol. Neonate* 43, 191-197
Caspari, P.G., Gilson, K. & Harris, P. (1975) *Cardiovasc, Res.* 9, 187-189
Cederbaum, A.I., Madhavan, T.V. & Rubin, E. (1976) *Biochem. Pharmacol.* 25, 1285-1292
Chalk, P.A., Higham, F.C., Caswell, A.H. & Bailey, E. (1983) *Int. J. Biochem.* 15, 531-538
Cham, B.E. & Knowles, B.R. (1976) *J. Lipid Res.* 17, 176-181
Chaudry, I.H. (1982) *Yale J. Biol. Med.* 55, 1-10
Chen, R.F. (1967) *J. Biol. Chem.* 242, 173-181
Christiansen, R.Z. (1978) *Biochim. Biophys. Acta* 530, 314-324
Christiansen, R.Z., Osmundsen, H., Borrebaek, B. & Bremer, J. (1978) *Lipids* 13, 487-491
Christie, K.N. & Stoward, P.J. (1979) *J. Histochem. Cytochem.* 27, 814-819
Christophersen, B.O. & Christiansen, R.Z. (1975) *Biochim. Biophys. Acta* 388, 402-412
Clarke, P.R.H. & Bieber, L.L.(1981) *J. Biol. Chem.* 256, 9861-9868
Close, R.I. (1972) *Physiol. Rev.* 52, 129-197
Conneely, O.H., Headon, D., Olson, C.D., Ungar, F. & Dempsey, M.E. (1983) *Fed. Proc.* 42, 1837 (Abstr.)
Connock, M.J. & Perry, S.R. (1983) *Biochem. Int.* 6, 545-551
Cook, G.A., Otto, D.A. & Cornell, N.W. (1980) *Biochem. J.* 192, 955-958
Crass, M.F. (1977) *Fed. Proc.* 36, 1995-1999
Crass, M.F., Shipp, J.C. & Pieper, G.M. (1975) *Am. J. Physiol.* 228, 618-627
Creese, R., D'Silva, J.L. & Shaw, D.M. (1962) *J. Physiol. (London)* 162, 44-53
Cryer, A. & Jones, H.M. (1978) *Biochem. J.* 174, 447-451
Dahlberg, E., Snochowski, M. & Gustafsson, J.-A. (1980) *Anal. Biochem.* 106, 380-388
Daum, H.A. & Dempsey, M.E. (1979) *Fed. Proc.* 38, 311 (Abstr.)

Daum, H.A. & Dempsey, M.E. (1980) *Fed. Proc.* 39, 1643
David, H., Meyer, R., Marx, I., Guski, H. & Wenzelides, K. (1979) *J. Mol. Cell. Cardiol.* 11, 631-638
Davies, C.T.H., Edwards, R.H.T. & Halliday, D. (1980) *J. Physiol.* 305, 898-908
Davies, K.J.A., Packer, L. & Brooks, G.A. (1981) *Arch. Biochem. Biophys.* 209, 539-554
De Brabander, H.F. & Verbeke, R. (1981) *Anal. Biochem.* 110, 240-241
De Duve, C. (1983) *Sci. Amer.* 243, no. 4, 52-62
De Grella, R.F. & Light, R.J. (1980 a) *J. Biol. Chem.* 255, 9731-9738
De Grella, R.F. & Light, R.J. (1980 b) *J. Biol. Chem.* 255, 9739-9745
Dempsey, M.E. (1974) *Annu. Rev. Biochem.* 43, 967-990
Dempsey, M.E., Rucker, R.D., Olson, C.D. & Buchwald, H. (1980) *Circulation* 62 III, 78 (Abstr.)
Dempsey, M.E., McCoy, K.E., Baker, H.N., Dimitriadou-Vafiadou, A., Lorsbach, T. & Howard, J.B. (1981) *J. Biol. Chem.* 256, 1867-1873
Dole, V.P. & Meinertz, H. (1960) *J. Biol. Chem.* 235, 2595-2599
Dow, J.W., Harding, N.G.L. & Powell, T. (1981 a) *Cardiovasc. Res.* 15, 483-514
Dow, J.W., Harding, N.G.L. & Powell, T. (1981 b) *Cardiovasc. Res.* 15, 549-579
Dyfverman, A. & Sjovall, J. (1978) *Anal. Lett.* B 11, 485-499
Eaton, P. & Steinberg, D. (1961) *J. Lipid Res.* 2, 376-382
Ellingboe, J., Nystrom, E. & Sjovall, J. (1970) *J. Lipid Res.* 11, 266-273
Engel, P.C. (1978) *Biochem. Soc. Trans.* 6, 76-80
Engel, A.G. & Angelini, C. (1973) *Science* 179, 899-903
Essén, B. (1977) *Ann. N.Y. Acad Sci.* 301, 30-44
Essén, B. (1978) *Acta Physiol. Scand* Suppl. 454, pp. 7-32
Fainaru, M., Schaeffer, Z. & Deckelbaum, R. (1981) *Prep. Biochem.* 11, 273-290
Farmer, B.B., Mancina, M., Williams, E.S. & Watanabe, A.M. (1983) *Life Sci.* 33, 1-18
Feuvray, D. & Plouet, J. (1981) *Circ. Res.* 48, 740-747
Fleischner, G., Meijer, D.K.F., Levine, W.G., Gatmaitan, Z., Gluck, R. & Arias, I.M. (1975) *Biochem. Biophys. Res. Commun.* 67, 1401-1407
Fleming, P.J. & Haigre, A.K. (1973) *Biochem. Biophys. Res. Commun.* 55, 743-751
Foster, P.C. & Bailey, E. (1976) *Biochem. J.* 154, 49-56
Fournier, N.C., Geoffroy, M. & Deshusses, J. (1978) *Biochim. Biophys. Acta* 533, 457-464
Fournier, N.C., Zuker, M., Williams, R.E. & Smith, I.C.P. (1983 a) *Biochemistry* 22, 1863-1872
Fournier, N.C. & Rahim, M.H. (1983 b) *J. Biol. Chem.* 258, 2929-2933
Frangakis, C.J., Bahl, J.J., McDaniel, H. & Bressler, R. (1980) *Life Sci.* 27, 815-825
Frayn, K.N. & Maycock, P.F. (1980) *J. Lipid Res.* 21, 139-144
Fritz, I.B. & Kaplan, E. (1960) *Am. J. Physiol.* 198, 39-44
Fritz, I.B., Davis, D.G., Holtrop, R.H. & Dundee, H. (1958) *Am. J. Physiol.* 194, 379-386
Fulco, A.J. (1967) *J. Biol. Chem.* 242, 3608-3616
Garland, P.B. & Randle, P.J. (1963) *Nature* 192, 381-382
Garthwaite, S.M., Morgan, R.F. & Meyer, D.K. (1979) *Proc. Soc. Exp. Biol. Med.* 160, 401-404
Glatz, J.F.C. & Veerkamp, J.H. (1982 a) *Biochim. Biophys. Acta* 711, 327-335
Glatz, J.F.C. & Veerkamp, J.H. (1982 b) *Biochim. Biophys. Acta* 713, 230-239

Glatz, J.F.C. & Veerkamp, J.H. (1983 a) *Anal. Biochem.* 132, 89-95
Glatz, J.F.C. & Veerkamp, J.H. (1983 b) *J. Biochem. Biophys. Meth.* 8, 57-61
Glatz, J.F.C., Wagenmakers, A.J.M., Veerkamp, J.H. & Van Moerkerk, H.T.B. (1983 a) *Biochem. Pharmacol.* 32, 2489-2493
Glatz, J.F.C., Baerwaldt, C.C.F., Veerkamp, J.H. & Kempen, H.J.M. (1983 b) *J. Biol. Chem.*, submitted for publication
Glatz, J.F.C., Jacobs, A.E.M. & Veerkamp, J.H. (1983 c) *J. Mol. Cell. Cardiol.*, submitted for publication
Gloster, J. & Harris, P. (1977) *Biochem. Biophys. Res. Commun.* 74, 506-513
Goldberg, A.L., Martel, S.B. & Kushmerick, M.J. (1975) *Meth. Enzymol.* 39, 82-94
Gollnick, P.D. (1977) *Ann. N.Y. Acad. Sci.* 301, 64-71
Gould, R.P. (1973) in *Structure and Function of Muscle* (Bourne, G.H., Ed), Vol. II, pp. 199-221, Acad. Press, New York
Grabowski, G., McCoy, K.E., Williams, G.C., Dempsey, M.E. & Hanson, R.F. (1976) *Biochim. Biophys. Acta* 441, 380-390
Gregersen, N. & Ingerslev, J. (1979) *Acta Paediatr. Scand.* 68, 677-681
Grimmer, G., Glaser, A., Oertel, H., Voigt, K.D. & Apostolakis, M. (1963) *Hoppe-Seyler's Z. Physiol. Chemie* 333, 232-241
Grinstead, G.F., Trzaskos, J.M., Billheimer, J.T. & Gaylor, J.L. (1983) *Biochim. Biophys. Acta* 751, 41-51
Grosso, D.S., Frangakis, C.J., Carlson, E.C. & Bressler, R. (1977) *Prep. Biochem.* 7, 383-401
Hagenfeldt, L. (1968) *Archiv. Kemi* 29, 57-62
Hagenfeldt, L. (1975) *Fed. Proc.* 34, 2246-2249
Hagenfeldt, L. (1979) *Diabetes* 28, 66-70
Hallman, M., Mäenpää, P. & Hassinen, I. (1972) *Experientia* 28, 1408-1410
Hamosh, M., Simon, M.R., Canter, H. & Hamosh, P. (1978) *Pediat. Res.* 12, 1132-1136
Hand, A.R. (1974) *J. Histochem. Cytochem.* 22, 207-209
Hansford, R.G. (1978) *Biochem. J.* 170, 285-295
Hansford, R.G. & Castro, F. (1982) *Mech. Ageing Dev.* 19, 191-201
Hanson, R.W. & Ballard, F.J. (1968) *J. Lipid Res.* 9, 667-668
Harris, P., Gloster, J. & Ward, B.J. (1980) *Arch. Mal. Cœur* 73, 593-598
Haunerland, N. & Spener, F. (1980) *Hoppe-Seyler's Z. Physiol. Chemie* 361, 262 (Abstr.)
Havel, R.J. & Kane, J.P. (1973) *Annu. Rev. Pharmacol.* 13, 287-308
Haworth, R.A., Hunter, D.R. & Berkoff, H.A. (1980) *J. Mol. Cell. Cardiol.* 12, 715-723
Hermansen, L. (1979) *Bull. Europ. Physiopath.* 15, 229-238
Higgins, A.J., Morville, M., Burges, R.A. & Blackburn, K.J. (1981) *Biochem. Biophys. Res. Commun.* 100, 291-296
Hirakow, R., Gotoh, T. & Watanabe, T. (1980) *Acta Anat.* 108, 144-152
Ho, R.J. (1970) *Anal. Biochem.* 36, 105-113
Hoppel, C.L. (1982) *Fed. Proc.* 41, 2853-2857
Hooker, A.M. & Baldwin, K.M. (1979) *Am. J. Physiol.* 236, C 66-69
Hryb, D.J. & Hogg, J.F. (1979) *Biochem. Biophys. Res. Commun.* 87, 1200-1206
Hull, F.E., Waugh, R.A. & Malone, M. (1972) *Arch. Biochem. Biophys.* 149, 69-90
Hülsmann, W.C., Stam, H. & Breeman, W.A.P. (1981) *Biochem. Biophys. Res. Commun.* 102, 440-448
Hülsmann, W.C., Stam. H. & Maccari, F. (1982) *Biochim. Biophys. Acta* 713, 39-45
Idell-Wenger, J.A. (1981) *J. Biol. Chem.* 256, 5597-5603

Idell-Wenger, J.A. & Neely, J.R. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J.M., Gotto, A.M. & Ontko, J.A., Eds), pp. 269-284, Williams & Wilkins Co., Baltimore, MD
Idell-Wenger, J.A., Grotyohann, L.W. & Neely, J.R. (1978) *J. Biol. Chem.* 253, 4310-4318
Idell-Wenger, J.A., Grotyohann, L.W. & Neely, J.R. (1982) *J. Mol. Cell. Cardiol.* 14, 413-417
Inestrosa, N.C., Bronfmann, M. & Leighton, F. (1980) *Biochem. Biophys. Res. Commun.* 95, 7-12
Iritani, N., Fukuda, E. & Inoguchi, K. (1980) *J. Nutr. Sci. Vitaminol.* 26, 271-277
Jolly, W.W., Wilhelm, D.D. & Harris, R.A. (1979) *J. Mol. Cell. Cardiol.* 11, 485-500
Jones, N.L., Heigenhauser, G.J.F., Kuksis, A., Matsos, C.G., Sutton, J.R. & Toews, C.J. (1980) *Clin. Sci.* 59, 469-478
Kam, W., Kumaran, K. & Landau, B.R. (1978) *J. Lipid Res.* 19, 591-600
Katz, A.M. & Messineo, F.C. (1982) *J. Mol. Cell. Cardiol.* 14, Suppl. 3, 119-122
Kawamura, N. & Kishimoto, Y. (1981) *J. Neurochem.* 36, 1786-1791
Kawashima, Y., Nakagawa, S. & Kozuka, H. (1982) *J. Pharm. Dyn.* 5, 771-779
Kaijser, L. (1982) in *Metabolic Risk Factors in Ischemic Cardiovascular Disease* (Carlson, L.A. & Pernow, B., Eds), pp. 149-158, Raven Press, New York
Kelly, A.M. & Rubinstein, N.A. (1980) *Nature* 288, 266-269
Kempen, H.J.M., Vos-Van Holstein, M.P.M. & De Lange, J. (1982) *J. Lipid Res.* 23, 823-830
Kempen, H.J.M., Vos-Van Holstein, M.P.M. & De Lange, J. (1983 a) *J. Lipid Res.* 24, 316-323
Kempen, H.J.M., Glatz, J.F.C., De Lange, J. & Veerkamp, J.H. (1983 b) *Biochem. J.*, in press
Kerner, J. & Bieber, L.L. (1982) *Biochem. Med.* 28, 204-209
Ketterer, B., Ross-Mansell, P. & Whitehead, J.K. (1967) *Biochem. J.* 103, 316-324
Ketterer, B., Tipping, E., Hackney, J.F. & Beale, D. (1976) *Biochem. J.* 155, 511-521
Keuper, H.J.K., Klein, R.A. & Spener, F. (1983) *Chem. Phys. Lipids* 32, 153-164
Kimura, T., Maji, T. & Ashida, K. (1970) *J. Nutr.* 100, 691-697
Kinnula, V.L. & Hassinen, I.E. (1977) *Acta Physiol. Scand.* 99, 462-466
Kloosterboer, H.J., Stoker-De Vries, S.A., Hulstaert, C.E. & Hommes, F.A. (1979) *Biol. Neonate* 35, 106-112
Kragh-Hansen, U. (1981) *Pharmacol. Rev.* 33, 17-53
Krahling, J.B., Gee, R., Gauger, J.A. & Tolbert, N.E. (1979) *J. Cell. Physiol.* 101, 375-390
Kramer, J.K.G. & Hulan, H.W. (1978) *J. Lipid Res.* 19, 103-106
Krieger, D.A., Tate, C.A., McMillin-Wood, J. & Booth, F.W. (1980) *J. Appl. Physiol.* 48, 23-28
Krisans, S.K., Mortensen, R.M. & Lazarow, P.B. (1980) *J. Biol. Chem.* 255, 9599-9607
Krstić, R.V. (1978) *Die Gewebe des Menschen und der Säugetieren*, Springer-Verlag, Berlin
Kupriyanov, V.V., Elizarova, G.V. & Saks, V.A. (1981) *Biokhimiya* 46, 930-941
Kurup, C.K.R., Aithal, H.N. & Ramasarma, T. (1970) *Biochem. J.* 116, 773-779

Laemmli, U.K. (1970) *Nature* 227, 680-685
Laker, M.E. & Mayes, P.A. (1979) *Biochem. Pharmacol.* 28, 2813-2829
Lang, C.A. (1965) *Biochem. J.* 95, 365-371
Langer, T. & Levy, R.J. (1968) *New Engl. J. Med.* 279, 856-858
Lassen, N.A., Parving, H.H. & Rossing, N. (1974) *Microvascular Res.* 7, i-iv
Latipää, P.M., Hiltunen, J.K., Peuhkurinen, K.J. & Hassinen, I.E. (1983) *Biochim. Biophys. Acta* 752, 162-171
Lazarow, P.B. (1977) *Science* 197, 580-581
Lazarow, P.B. (1978) *J. Biol. Chem.* 253, 1522-1528
Lazarow, P.B. (1981 a) *Arch. Biochem. Biophys.* 206, 342-345
Lazarow, P.B. (1981 b) *Meth. Enzymol.* 72, 315-319
Lazarow, P.B. (1982) in *Metabolic Compartmentation* (Sies, H., Ed), pp. 317-330, Acadademic Press, New York
Lazarow, P.B. & De Duve (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 2043-2046
Lee, H.-J. & Wilson, I.B. (1971) *Biochim. Biophys. Acta* 242, 519-522
Levi, A.J., Gatmaitan, Z. & Arias, I.M. (1969) *J. Clin. Invest.* 48, 2156-2167
Lin, M.-Y. & Kako, K.J. (1975) *J. Mol. Cell. Cardiol.* 7, 577-590
Linder, C., Chernick, S.S., Fleck, T.R. & Scow, R.O. (1976) *Am. J. Physiol.* 231, 860-864
Lipsky, N.G. & Pedersen, P.L. (1982) *J. Biol. Chem.* 257, 1473-1481
Lithell, H., Boberg, J., Hellsing, K., Lundqvist, G. & Vessby, B. (1978) *Atherosclerosis* 30, 89-94
Lithell, H., Orlander, J. & Schele, R. (1979) *Acta Physiol. Scand.* 107, 257-261
Lockwood, E.A. & Bailey, E. (1970) *Biochem. J.* 120, 49-54
Lopes-Cardozo, M. & Van den Bergh, S.G. (1972) *Biochim. Biophys. Acta* 283, 1-15
Lopes-Cardozo, M. & Van den Bergh, S.G. (1974) *Biochim. Biophys. Acta* 357, 43-52
Lopes-Cardozo, M., Klazinga, W. & Van den Bergh, S.G. (1978) *Eur. J. Biochem.* 83, 635-640
Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1953) *J. Biol. Chem.* 193, 265-275
Lundholm, K., Bylund, A.-C., Holm, J., Smeds, S. & Scherstén, T. (1975) *Europ. Surg. Res.* 7, 65-82
Lunzer, M.A., Manning, J.A. & Ockner, R.K. (1977) *J. Biol. Chem.* 252, 5483-5487
Mackerer, C.R. (1977) *Biochem. Pharmacol.* 26, 2225-2230
Mackie, B.G., Dudley, G.A., Kaciuba-Uscilko, H. & Terjung, R.L. (1980) *J. Appl. Physiol.* 49, 851-855
Mannaerts, G.P., Thomas, J., Debeer, L.J., McGarry, J.D. & Foster, D.W. (1978) *Biochim. Biophys. Acta* 529, 201-211
Mannaerts, G.P., Debeer, L.J., Thomas, J. & De Schepper, P.J. (1979) *J. Biol. Chem.* 254, 4585-4595
Markwell, M.A.K., McGroarty, E.J., Bieber, L.L. & Tolbert, N.E. (1973) *J. Biol. Chem.* 248, 3426-3432
Markwell, M.A.K., Tolbert, N.E. & Bieber, L.L. (1976) *Arch. Biochem. Biophys.* 176, 479-488
Markwell, M.A.K., Bieber, L.L. & Tolbert, N.E. (1977) *Biochem. Pharmacol.* 26, 1697-1702
Masters, C.J. & Holmes, R.S. (1977) *Physiol. Rev.* 57, 816-881
Masters, C.J. & Holmes, R.S. (1979) *Trends Biochem. Sci.* 4, 233-236
Masoro, E.J. (1967) *J. Biol. Chem.* 242, 1111-1114

Matlib, M.A., Rebman, D., Ashraf, M., Rouslin, W. & Schwartz, A. (1981) *J. Mol. Cell. Cardiol.* 13, 163-170
Matsushita, Y., Umeyama, H. & Moriguchi, I. (1977) *Chem Pharm. Bull.* 25, 647-652
McGarry, J.D. & Foster, D.W. (1981) *Biochem. J.* 200, 217-223
McGarry, J.D., Robles-Valdes, C. & Foster, D.W. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 4385-4388
McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1977) *J. Clin. Invest.* 60, 265-270
McGarry, J.D., Leatherman, G.F. & Foster, D.W. (1978 a) *J. Biol. Chem.* 253, 4128-4136
McGarry, J.D., Takahayashi, Y. & Foster, D.W. (1978 b) *J. Biol. Chem.* 253, 8294-8300
McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1978 c) *Biochim. Biophys. Acta* 530, 305-313
McGarry, J.D., Mills, S.E., Long, C.S. & Foster, D.W. (1983) *Biochem. J.* 214, 21-28
McGuire, D.M., Olson, C.D., Towle, H.D. & Dempsey, M.E. (1983) *Fed. Proc.* 42, 1837 (Abstr.)
McLane, J.A., Fell, R.D., McKay, R.H., Winder, W.W., Brown, E.B. & Holloszy, J.O. (1981) *Am. J. Physiol.* 241, C 47-54
McMillin-Wood, J. (1975) *J. Biol. Chem.* 250, 3062-3066
Menon, N.K. & Dhopeshwarkar, G.A. (1983) *Biochim. Biophys. Acta* 751, 14-20
Middleton, B. (1978) *Biochem. Soc. Trans.* 6, 80-83
Miller, N.E., Mjøs, O.D. & Oliver, M.F. (1976) *Clin. Sci. Molec. Med.* 51, 209-213
Mishkin, S. & Turcote, R. (1974) *Biochem. Biophys. Res. Commun.* 57, 918-926
Mishkin, S. & Halperin, M.L. (1977) *Adv. Exp. Med. Biol.* 92, 583-605
Mishkin, S., Stein, L., Gatmaitan, Z. & Arias, I.M. (1972) *Biochem. Biophys. Res. Commun.* 47, 997-1003
Mishkin, S., Stein, L., Fleischner, G., Gatmaitan, Z., Gluck, R. & Arias, I.M. (1975) *Am. J. Physiol.* 228, 1634-1640
Mishkin, S., Morris, H.P., Murthy, P.V.N. & Halperin, M.L. (1977) *J. Biol. Chem.* 252, 3626-3628
Mitchell, P. (1977) *Annu. Rev. Biochem.* 46, 996-1005
Moore, K.H., Radloff, J.F., Koen, A.E. & Hull, F.E. (1982) *J. Mol. Cell. Cardiol.* 14, 451-459
Morand, O., Fibach, E. & Gatt, S. (1982) *Biochim. Biophys. Acta* 693, 143-150
Morin, R.J., Brun, M.J. & Srikantaiah, M.V. (1982) *Lipids* 17, 507-510
Morrison, W.R. & Smith, L.M. (1964) *J. Lipid Res.* 5, 600-608
Morrow, F.D. & Martin, R.J. (1980) *Fed. Proc.* 39, 1124 (Abstr.)
Morrow, F.D. & Martin, R.J. (1983) *J. Lipid Res.* 24, 324-331
Morrow, F.D., Allen, C.E. & Martin, R.J. (1979) *Fed. Proc.* 38, 280 (Abstr.)
Morton, R.K. (1955) *Meth. Enzymol.* 1, 25-51
Nakano, N.I., Shimamori, Y. & Nakano, M. (1983) *Anal. Biochem.* 129, 64-71
Nakata, K. (1977) *Jap. Circulation J.* 41, 1237-1250
Neat, C.E., Thomassen, M.S. & Osmundsen, H. (1981) *Biochem. J.* 196, 149-159
Neely, J.R., Whitmer, K.M. & Mochizucki, S. (1976) *Circ. Res.* 38, I 22-30
Neely, J.R. & Morgan, H.E. (1974) *Annu. Rev. Physiol.* 36, 413-459
Neptune, E.M., Sudduth, H.C. & Foreman, D.R. (1959) *J. Biol. Chem.* 234, 1659-1666
Nestel, P.J. & Grundy, S.M. (1976) *Metabolism* 25, 1259-1267

Newsholme, E.A., Sugden, P.H. & Williams, T. (1977) *Biochem. J.* 166, 123-129
Normann, P.T., Norseth, J. & Flatmark, T. (1983) *Biochim. Biophys. Acta* 752, 474-481
Norseth, J. (1980) *Biochim. Biophys. Acta* 617, 183-191
Norseth, J. & Thomassen, M.S. (1983) *Biochim. Biophys. Acta* 751, 312-320
Norseth, J., Normann, P.T. & Flatmark, T. (1982) *Biochim. Biophys. Acta* 719, 569-579
Novák, E., Drummond, G.I., Skála, J. & Hahn, P. (1972) *Arch. Biochem. Biophys.* 150, 511-518
Ockner, R.K. & Manning, J.A. (1974) *J. Clin. Invest.* 54, 326-338
Ockner, R.K. & Manning, J.A. (1976) *J. Clin. Invest.* 58, 632-641
Ockner, R.K., Manning, J.A., Poppenhausen, R.B. & Ho, W.K.L. (1972) *Science* 177, 56-58
Ockner, R.K., Burnett, D.A., Lysenko, N. & Manning, J.A. (1979) *J. Clin. Invest.* 64, 172-181
Ockner, R.K., Lysenko, N., Manning, J.A., Monroe, S.E. & Burnett, D.A. (1980) *J. Clin. Invest.* 65, 1013-1023
Ockner, R.K., Manning, J.A. & Kane, J.P. (1982) *J. Biol. Chem.* 257, 7872-7878
Ockner, R.K., Weisiger, R.A. & Gollan, J.L. (1983) *Am. J. Physiol.* 245, G 13-18
O'Doherty, P.J.A. & Kuksis, A. (1975) *FEBS Lett.* 60, 256-258
O'Hare, M.J. & Nice, E.C. (1979) *J. Chromatogr.* 171, 209-226
Okano, G., Matsuzaka, H. & Shimojo, T. (1980) *Biochim. Biophys. Acta* 619, 167-175
Okano, G. & Shimojo, T. (1982) *Biochim. Biophys. Acta* 710, 122-127
Olivetti, G., Anversa, P. & Loud, A.V. (1980) *Circ. Res.* 46, 503-512
Ontko, J.A. (1972) *J. Biol. Chem.* 247, 1788-1800
Ontko, J.A. & Johns, M.L. (1980) *Biochem. J.* 192, 959-962
Oram, J.F., Bennetch, S.L. & Neely, J.R. (1973) *J. Biol. Chem.* 248, 5299-5309
Oram, J.F., Wenger, J.I. & Neely, J.R. (1975) *J. Biol. Chem.* 250, 73-78
Oscai, L.B., Caruso, R.A. & Wergeles, A.C. (1982) *J. Appl. Physiol.* 52, 1059-1063
Osmundsen, H. (1981) *Meth. Enzymol.* 72, 306-314
Osmundsen, H., Neat, C.E. & Norum, K.R. (1979) *FEBS Lett.* 99, 292-296
Osmundsen, H., Neat, C.E. & Borrebaek, B. (1980 a) *Int. J. Biochem.* 12, 625-630
Osmundsen, H., Christiansen, R.Z. & Bremer, J. (1980 b) in *Carnitine Biosynthesis, Metabolism and Function* (Frenkel, R.A. & McGarry, J.D., Eds), pp. 127-140, Academic Press, New York
Osumi, T. & Hashimoto, T. (1978) *Biochem. Biophys. Res. Commun.* 83, 479-485
Osumi, T, Hashimoto, T. & Ui, N. (1980) *J. Biochem.* 87, 1735-1746
Palmer, J.W., Tandler, B. & Hoppel, C.L. (1977) *J. Biol. Chem.* 252, 8731-8739
Palmer, W.K., Caruso, R.A. & Oscai, L.B. (1981) *Biochem. J.* 198, 159-166
Pande, S.V. (1971) *J. Biol. Chem.* 246, 5384-5390
Pande, S.V. & Parvin, R. (1980) *Biochim. Biophys. Acta* 617, 363-370
Paris, S., Samuel, D., Jacques, Y., Gache, C., Franchi, A. & Ailhaud, G. (1978) *Eur. J. Biochem.* 83, 235-243
Paul, H.S. & Adibi, S.A. (1979) *J. Clin. Invest.* 64, 405-412
Paul, H.S. & Adibi, S.A. (1980) *J. Clin. Invest.* 65, 1285-1293

Pearce, F.J. & Connett, R.J. (1980) *Am. J. Physiol.* 238, C 149-159
Pearce, F.J., Forster,J., De Leeuw, G., Williamson, J.R. & Tutwiler, G.F. (1979) *J. Mol. Cell. Cardiol.* 11, 893-915
Peter, J.B., Barnard, R.J., Edgerton, V.R., Gillespie, C.A. & Stempel, K.E. (1972) *Biochemistry* 11, 2627-2633
Rabinowitz, J.L. & Herscher, E.G. (1974) *Arch. Biochem. Biophys.* 161, 621-627
Rakusan, K. (1980) in *Hearts and Heart-like organs*, Vol. I, pp. 301-347, Academic Press, New York
Rakusan, K., Raman, S., Layberry, R. & Korecky, B. (1978) *Circ. Res.* 42, 212-218
Rasheed, B.K.A., Chabra, J. & Kurup, C.K.R. (1980) *Biochem. J.* 190, 191-198
Rebouche, C.J. (1977) *Biochim. Biophys. Acta* 471, 145-155
Reddy, J.K., Warren, J.R., Reddy, M.K. & Lalwani, N.D. (1982) *Ann. N.Y. Acad. Sci.* 386, 81-108
Reichen, J. & Berk, P.D. (1979) *Biochem. Biophys. Res. Commun.* 91, 484-489
Reitman, J., Baldwin, K.M. & Holloszy, J.O. (1973) *Proc. Soc. Exp. Biol. Med.* 142, 628-631
Renaud, G., Foliot, A. & Infante, R. (1978) *Biochem. Biophys. Res. Commun.* 80, 327-334
Rennie, M.J. & Holloszy, J.O. (1977) *Biochem. J.* 168, 161-170
Rennie, M.J., Edwards, R.H.T., Krywawych, S., Davies, C.T.M., Halliday, D., Waterlow, J.C. & Millward, D.J. (1981) *Clin. Sci.* 61, 627-639
Reynafarje, B. (1963) *J. Lab. Clin. Med.* 61, 138-145
Righetti, P.G. & Caravaggio, T. (1976) *J. Chromatogr.* 127, 1-28
Righetti, P.G., Tudor, G. & Ek, K. (1981) *J. Chromatogr.* 220, 115-194
Ritter, M.C. & Dempsey, M.E. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 265-269
Robles-Valdes, C., McGarry, J.D. & Foster, D.W. (1976) *J. Biol. Chem.* 251, 6007-6012
Rodwell, V.W., Nordstrom, J.L. & Mitschelen, J.J. (1976) *Adv. Lipid Res.* 14, 1-74
Rogiers, V. (1981) *J. Lipid Res.* 22, 1-6
Rolph, T.P., Jones, C.T. & Parry, D. (1982) *Am. J. Physiol.* 243, H 87-93
RÜstow, B., Hodi, J., Kunze, D., Reichmann, G. & Egger, E. (1978) *FEBS Lett.* 95, 225-228
Rüstow, B., Kunze, D., Hodi, J. & Egger, E. (1979) *FEBS Lett.* 108, 469-472
Rüstow, B., Risse, S. & Kunze, D. (1982) *Acta Biol. Med. Germ.* 41, 439-445
Saggerson, E.D. & Carpenter, C.A. (1981) *FEBS Lett.* 129, 229-232
Saks, V.A., Rosenshtraukh, L.V., Smirnov, V.N. & Chazov, E.I. (1978) *Can. J. Physiol. Pharmacol.* 36, 691-706
Saks, V.A., Kupriyanov, V.V., Elizarova, G.V. & Jacobus, W.E. (1980) *J. Biol. Chem.* 255, 755-763
Sallee, V.L. (1974) *J. Lipid Res.* 15, 56-64
Samuel, D., Paris, S. & Ailhaud, G. (1976) *Eur. J. Biochem.* 64, 583-595
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* 51, 660-672
Scholte, H.R. (1973 a) *Biochim. Biophys. Acta* 305, 413-427
Scholte, H.R. (1973 b) *Biochim. Biophys. Acta* 330, 283-293
Schonfeld, G. & Kipnis, D.M. (1968) *Am. J. Physiol.* 215, 513-522
Schuder, S., Wittenberg, J.B., Haseltine, B. & Wittenberg, B.A. (1979) *Anal. Biochem.* 92, 473-481
Schwartzmann, L.I. & Brown, J. (1960) *Am. J. Physiol.* 199, 235-237
Scow, R.O., Blanchette-Mackie, E.J. & Smith, L.C. (1980) *Fed. Proc.* 39, 2610-2617

Seifert, J. (1980) *Arch. Biochem. Biophys.* 201, 194-198
Seraydarian, M.W. (1982) *Trends Pharmacol. Sci.* 3, 393-395
Severson, D.L. (1979) *Can. J. Physiol. Pharmacol.* 57, 923-937
Shapiro, D.J. & Rodwell, V.W. (1969) *Biochem. Biophys. Res. Commun.* 37, 867-872
Shepherd, D. & Garland, P.B. (1969) *Meth. Enzymol.* 13, 11-13
Shindo, Y. & Hashimoto, T. (1978) *J. Biochem.* 84, 1177-1181
Shumate, J.B. & Choksi, R.M. (1981) *Biochem. Biophys. Res. Commun.* 100, 978-981
Shumate, J.B., Carroll, J.E., Brooke, M.H. & Choksi, R.M. (1982) *Muscle & Nerve* 5, 226-231
Skrede, S. & Halvorsen, O. (1979) *Eur. J. Biochem.* 98, 223-229
Small, G.M., Brolly, D. & Connock, M.J. (1980) *Life Sci.* 27, 1743-1751
Smith, L. (1955) in *Methods of Biochemical Analysis* (Glick, D., Ed), Vol II, pp. 427-434, Wiley, New York
Smith, P.B. (1980) *Biochim. Biophys. Acta* 628, 19-25
Smith, D.M. & Sugden, P.H. (1983) *Biochem. J.* 214, 497-502
Smith, F.M.M., Dils, R.R. & Gurr, M.I. (1983) *Biochem. Soc. Trans.* 11, 308 (Abstr.)
Sogami, M. & Foster, J.F. (1968) *Biochemistry* 7, 2172-2182
Song, M.-K.H. & Dempsey, M.E. (1981) *Arch. Biochem. Biophys.* 211, 523-529
Spector, A.A., Steinberg, D. & Tanaka, A. (1965) *J. Biol. Chem.* 240, 1032-1041
Spector, A.A. (1971) *Progr. Biochem. Pharmacol.* 6, 130-176
Spector, A.A. (1975) *J. Lipid Res.* 16, 165-179
Spector, A.A. & Santos, E.C. (1973) *Ann. N.Y. Acad. Sci.* 226, 247-258
Spector, A.A. & Fletcher, J.E. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J.M., Gotto, A.M. & Ontko, J.A., Eds), pp. 229-249, Williams & Wilkins Co., Baltimore, MD
Spence, J.T. & Gaylor, J.L. (1977) *J. Biol. Chem.* 252, 5852-5858
Srikantaiah, M.V., Hansbury, E., Loughran, E.D. & Scallen, T.J. (1976) *J. Biol. Chem.* 251, 5496-5504
Stam, H. & Hülsmann, W.C. (1981) *Ann. Nutr. Metab.* 25, 11-19
Stankiewicz-Choroszucha, H. & Gôrski, J. (1978) *Experientia* 34, 357-358
Stanley, K.K. & Tubbs, P.K. (1974) *FEBS Lett.* 39, 325-328
Stanley, K.K. & Tubbs, P.K. (1975) *Biochem. J.* 150, 77-88
Stearns, S.B., Tepperman, H.M. & Tepperman, J. (1979) *J. Lipid Res.* 20, 654-662
Stein, L.B., Mishkin, S., Fleischner, G., Gatmaitan, Z. & Arias, I.M. (1976) *Am. J. Physiol.* 231, 1371-1376
Steinhardt, J., Leidy, J.G. & Mooney, J.P. (1972) *Biochemistry* 11, 1809-1817
Stockdale, F.E., Raman, N. & Baden, H. (1981) *Proc. Natl. Acad. Sci. U.S. A.* 78, 931-935
Stollman, Y.R., Gärtner, U., Theilmann, L., Ohmi, N. & Wolkoff, A.W. (1983) *J. Clin. Invest.* 72, 718-723
Stremmel, W., Potter, B.J. & Berk, P.D. (1983 a) *Biochim. Biophys. Acta* 756, 20-27
Stremmel, W., Kochwa, S. & Berk, P.D. (1983 b) *Biochem. Biophys. Res. Commun.* 112, 88-95
Sumbilla, C.M., Zielke, C.L., Reed, W.D., Ozand, P.T. & Zielke, H.R. (1981) *Biochim. Biophys. Acta* 675, 301-304
Suzue, G. & Marcel, Y.L. (1975) *Can. J. Biochem.* 53, 804-809
Taegtmeyer, H., Hems, R. & Krebs, H.A. (1980) *Biochem. J.* 186, 701-711

Takahashi, K., Odani, S. & Ono, T. (1982) *FEBS Lett.* 140, 63-66
Tamboli, A., O'Looney, P., Van der Maten, M. & Vahouny, G.V. (1983) *Biochim. Biophys. Acta* 750, 404-410
Thomas, J., Debeer, L.J., De Schepper, P.J. & Mannaerts, G.P. (1980) *Biochem. J.* 190, 485-494
Thorp, J.M. (1963) *J. Artherosclerosis* 3, 351-360
Thorp, J.M. & Waring, W.J. (1962) *Nature* 194, 948-949
Timson, B.F. (1982) *Growth* 46, 36-45
Tolbert, N.E. (1981) *Annu. Rev. Biochem.* 50, 133-157
Tomec, R.J. & Hoppel, C.L. (1975) *Arch. Biochem. Biophys.* 170, 716-723
Trulzsch, D. & Arias, I.M. (1981) *Arch. Biochem. Biophys.* 209, 433-440
Tutwiler, G.F. (1978) *Res. Commun. Chem. Pathol. Pharm.* 19, 541-544
Tutwiler, G.F. & Ryzlak, M.T. (1980) *Life Sci.* 26, 393-397
Ul-Haq, R., Christodoulides, L. Ketterer, B. & Shrago, E. (1982) *Biochim. Biophys. Acta* 713, 193-198
Ul-Haq, R., Shrago, E. & Farrell, P.M. (1983) *Fed. Proc.* 42, 1323 (Abstr.)
Vahouny, G.V., Wei, R.W., Tamboli, A. & Albert, E.N. (1979) *J. Mol. Cell. Cardiol.* 11, 339-357
Van der Vusse, G.J., Roemen, T.H.M. & Reneman, R.S. (1980) *Biochim. Biophys. Acta* 617, 347-352
Van der Vusse, G.J., Roemen, T.H.M., Flameng, W. & Reneman, R.S. (1983) *Biochim. Biophys. Acta* 752, 361-370
Van Hinsbergh, V.W.M. (1979) Thesis, University of Nijmegen
Van Hinsbergh, V.W.M., Veerkamp, J.H. & Van Moerkerk, H.T.B. (1978 a) *Arch. Biochem. Biophys.* 190, 762-771
Van Hinsbergh, V.W.M., Veerkamp, J.H. & Van Moerkerk, H.T.B. (1978 b) *Biochem. Med.* 20, 256-266
Van Hinsbergh, V.W.M., Veerkamp, J.H. & Bookelman, H. (1979) *J. Mol. Cell. Cardiol.* 11, 1245-1252
Van Hinsbergh, V.W.M., Veerkamp, J.H. & Van Moerkerk, H.T.B. (1980) *J. Neurol. Sci.* 47, 79-91
Van Tol, A. (1974) *Biochim. Biophys. Acta* 357, 14-23
Veerkamp, J.H. (1970) *Biochim. Biophys. Acta* 210, 267-275
Veerkamp, J.H. (1981) in *Mitochondria and Muscular Diseases* (Busch, H.F.M., Jennekens, F.G.I. & Scholte, H.R., Eds), pp. 29-50, Mefar, Beetsterzwaag, The Netherlands
Veerkamp, J.H. & Van Moerkerk, H.T.B. (1982) *Biochim. Biophys. Acta* 710, 252-255
Veerkamp, J.H., Van Moerkerk, H.T.B., Glatz, J.F.C. & Van Hinsbergh, V.W.M. (1983) *Biochim. Biophys. Acta*, in press
Vermeer, B.J., Reman, F.C. & Van Gent, C.M. (1979) *J. Invest. Dermatol.* 73, 303-305
Vignais, P.M., Gallagher, C.H. & Zabin, J. (1958) *J. Neurochem.* 2, 283-287
Villa-Moruzzi, E., Locci-Cubeddu, T. & Bergamini, E. (1979) *Growth* 43, 73-79
Vitorica, J., Cano, J., Satrústegui, J. & Machado, A. (1981) *Mech. Ageing Dev.* 16, 105-116
Voltti, H. & Hassinen, I.E. (1980) *Biochem. Pharmacol.* 29, 987-992
Wagenmakers, A.J.M. & Veerkamp, J.H. (1982) *Biochem. Med.* 28, 16-31
Wahren, J. (1979) *Diabetes*, Suppl. 28, 82-88
Walpurger, G. (1967) *Klin. Wochenschr.* 45, 239-244
Wang, H., Rasmussen, K., Vik-Mo, H., Mjøs, O.D. & Grendahl, H. (1979) *Acta Med. Scand.* 205, 299-301
Warner, M. & Neims, A.H. (1975) *Can. J. Physiol. Pharmacol.* 53, 493-500

Warshaw, J.B. (1972) *Dev. Biol.* 28, 537-544
Warshaw, J.B. & Terry, M.L. (1970) *J. Cell. Biol.* 44, 354-360
Warshaw, J.B. & Terry, M.L. (1976) *Dev. Biol.* 52, 161-166
Weis, H.J. & Dietschy, J.M. (1975) *Biochim. Biophys. Acta* 398, 315-324
Weisiger, R.A., Gollan, J.L. & Ockner, R.K. (1980) *Gastroenterology* 79, 1065 (Abstr.)
Weisiger, R.A., Gollan, J.L. & Ockner, R.K. (1981) *Science* 211, 1048-1051
Weisiger, R.A., Gollan, J.L. & Ockner, R.K. (1982) in *Progress in Liver Diseases* (Popper, H. & Schaffner, F., Eds), Vol. VII, pp. 71-85, Grune & Stratton, New York
Whereat, A.F. (1971) *Adv. Lipid Res.* 9, 119-159
Williams, E.J. & Foster, J.F. (1960) *J. Amer. Chem. Soc.* 82, 3741-3745
Willner, J., DiMauro, J., Eastwood, A., Hays, A., Rochi, F. & Lovelace, R. (1979) *J. Neurol. Sci.* 41, 235-246
Wilson, D.F., Nishiki, K. & Erecińska, M. (1981) *Trends Biochem. Sci.* 6, 16-19
Wittels, B. & Bressler, R. (1965) *J. Clin. Invest.* 44, 1639-1646
Witztum, J.W., Schonfeld, G. & Weidman, S.W. (1976) *J. Lab. Clin. Med.* 88, 1008-1018
Wolfe, R.G., Maxwell, C.V. & Nelson, E.C. (1978) *J. Nutr.* 108, 1621-1634
Wolkoff, A.W. & Chung, C.T. (1980) *J. Clin. Invest.* 65, 1152-1161
Wu-Rideout, M.Y.C., Elson, C. & Shrago, E. (1976) *Biochem. Biophys. Res. Commun.* 71, 809-816
Yokota, S. (1982) *Histochemistry* 74, 379-386

Parts of this thesis have been or will be published elsewhere:

1. Glatz, J.F.C. and J.H. Veerkamp
   *Postnatal development of palmitate oxidation and mitochondrial enzyme activities in rat cardiac and skeletal muscle.*
   Biochim. Biophys. Acta (1982) 711, 327-335
2. Glatz, J.F.C. and J.H. Veerkamp
   *Palmitate oxidation by intact preparations of skeletal muscle.*
   Biochim. Biophys. Acta (1982) 713, 230-239
3. Glatz, J.F.C. and J.H. Veerkamp
   *A radiochemical procedure for the assay of fatty acid binding by proteins.*
   Anal. Biochem. (1983) 132, 89-95
4. Glatz, J.F.C. and J.H. Veerkamp
   *Removal of fatty acids from serum albumin by Lipidex 1000 chromatography.*
   J. Biochem. Biophys. Meth. (1983) 8, 57-61
5. Glatz, J.F.C., A.J.M. Wagenmakers, J.H. Veerkamp and H.T.B. Van Moerkerk
   *Effect of clofibrate feeding on palmitate and branched-chain 2-oxo acid oxidation in rat liver and muscle.*
   Biochem. Pharmacol. (1983) 32, 2489-2493
6. Veerkamp, J.H., H.T.B. Van Moerkerk, J.F.C. Glatz and V.W.M. Van Hinsbergh
   *Incomplete palmitate oxidation in cell-free systems of rat and human muscles.*
   Biochim. Biophys. Acta (1983) in press
7. Kempen, H.J.M., J.F.C. Glatz, J. De Lange and J.H. Veerkamp
   *Concomitant increase in hepatic triacylglycerol biosynthesis and cytosolic fatty acid-binding protein content after feeding rats a cholestyramine-containing diet.*
   Biochem. J. (1983) in press
8. Glatz, J.F.C., C.C.F. Baerwaldt, J.H. Veerkamp and H.J.M. Kempen
   *Diurnal variation of cytosolic fatty acid-binding protein content and of palmitate oxidation in rat liver and heart.*
   J. Biol. Chem., submitted for publication
9. Glatz, J.F.C., A.E.M. Jacobs and J.H. Veerkamp
   *Fatty acid oxidation in human and rat heart. Comparison of cell-free and cellular systems.*
   J. Mol. Cell. Cardiol., submitted for publication
10. Glatz, J.F.C., C.C.F. Baerwaldt, A.M. Janssen and J.H. Veerkamp
    *Purification and characterization of fatty acid-binding protein from rat heart and liver.*
    Manuscript in preparation
11. Glatz, J.F.C. and J.H. Veerkamp
    *Fatty acid-binding proteins (mini-review).*
    Manuscript in preparation

# CURRICULUM VITAE

De auteur van dit proefschrift werd op 20 januari 1955 te Oss geboren. Na het behalen van het diploma HBS-B aan het St. Canisius College te Nijmegen in 1972 begon hij in datzelfde jaar de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Op 30 juni 1975 werd het kandidaatsexamen (S 2) afgelegd. Voor de doctoraalstudie werden twee hoofdvakstages gevolgd: biofysische chemie (Prof. Dr. C.W. Hilbers en Dr. P.J.M. Salemink) en biochemie, gedeeltelijk uitgevoerd in Nijmegen (Prof. Dr. J.H. Veerkamp en Dr. V.W.M. Van Hinsbergh) en aan de Rijksuniversiteit te Utrecht (Prof. Dr. K.W.A. Wirtz en Dr. B.J.H.M. Poorthuis). Daarnaast behaalde hij op 21 januari 1976 het diploma deskundigheid stralingshygiëne voor een C-laboratorium. Tijdens de doctoraalstudie verleende hij assistentie bij het opzetten en begeleiden van een radionuclidencursus voor chemici. Op 25 juni 1979 werd het doctoraalexamen afgelegd.

Van 1 augustus 1979 tot 1 augustus 1983 was hij als wetenschappelijk medewerker in dienst van de afdeling Biochemie, Medische Faculteit van de Katholieke Universiteit te Nijmegen. In deze functie verrichtte hij onder leiding van Prof. Dr. J.H. Veerkamp een onderzoek waarin diverse aspecten van het vetzuurmetabolisme in skelet- en hartspier werden bestudeerd, zoals in dit proefschrift is beschreven. Gedurende deze tijd leverde hij tevens een bijdrage aan het onderwijs aan medische en tandheelkundige studenten.

In februari 1983 werd hij door financiële steun van de Medische Faculteit in de gelegenheid gesteld werkbezoeken te brengen aan vijf laboratoria in de U.S.A.

# TOELICHTING

## VETZUUROXIDATIE IN SKELET- EN HARTSPIER

De spier speelt naast het skelet een belangrijke rol bij de houding en beweging van mens en dier. De voor het verrichten van spierarbeid benodigde energie wordt voornamelijk verkregen door verbranding van koolhydraten en vetten, waarbij de laatste de belangrijkste bijdrage leveren. Grote verschillen in spieractiviteit zijn mogelijk door de aanwezigheid van een uiterst flexibel mechanisme, dat op elk moment zorg draagt voor de juiste energievoorziening. Het spierweefsel omvat 40 tot 50% van het lichaamsgewicht en is daardoor ook zeer belangrijk voor de energiehuishouding van het lichaam. Onze kennis omtrent het verloop van de energievoorziening in de spier en de regulatie daarvan is beperkt, met name bij de mens. Bij het in dit proefschrift beschreven biochemisch onderzoek is gekeken naar de betekenis van vetzuren (uit vetten gesplitste voedingsstoffen) voor de energieproductie in skelet- en hartspier.

De verbranding van vetzuren tot koolstofdioxide ($CO_2$) en water vindt plaats in bepaalde organellen van de cel, de mitochondriën, die energielevering als belangrijkste functie hebben. Bij de bestudering van de vetzuurverbranding (vetzuuroxidatie) is het gebruik van intacte mitochondriën een vereiste. We kunnen gebruik maken van verschillende spierpreparaten, zoals zgn. cellulaire systemen (hele spieren en geïsoleerde spiercellen) en celvrije systemen (spierhomogenaten en geïsoleerde mitochondriën). Voor meting van de vetzuurverbranding wordt gebruik gemaakt van radioactief gemerkte vetzuren, waarbij de verbrandingssnelheid wordt berekend uit de vorming van radioactief gemerkte oxidatieproducten. Met deze methode zijn onder andere de eigenschappen vergeleken van de celvrije en de meer complexe cellulaire systemen, en ook de verschillen bestudeerd tussen skelet- en hartspierpreparaten

afkomstig van mens en rat. De hartspier heeft een hogere capaciteit voor vetzuurverbranding dan de onderzochte skeletspieren, die op hun beurt ook weer onderling verschillen in verbrandingscapaciteit. De bepalingsmethode kan gebruikt worden voor diagnostiek van spierziekten die het gevolg zijn van storingen in de vetstofwisseling.

De in de spier benodigde vetzuren worden door het bloed aangevoerd gebonden aan het eiwit albumine, aangezien ze slecht oplosbaar zijn in water. Door de spier wordt alleen het vetzuur opgenomen; het albumine blijft in het bloed achter. Hoe de vetzuren vervolgens in de spiercel naar de mitochondriën worden getransporteerd is nog niet duidelijk. Men neemt aan dat ook hierbij een eiwit is betrokken. Inderdaad heeft men in verschillende weefsels, waaronder de spier, een klein eiwit aangetoond dat vetzuren kan binden. Van dit vetzuurbindend eiwit is echter nog weinig bekend, met name in de spier. In dit proefschrift wordt een gevoelige methode beschreven voor de bepaling hiervan. Gebruikmakend van deze bepalingsmethode bleek dat zowel de hartspier als de lever van de rat een hoog vetzuurbindend eiwitgehalte hebben en tevens dat dit gehalte 's nachts tweemaal zo hoog is als overdag. Deze opmerkelijke resultaten doen vermoeden dat het vetzuurbindend eiwit een belangrijke rol vervult bij de stofwisseling van vetzuren door regulatie van de beschikbaarheid van vetzuren. Het is bekend dat in de hartspier hoge concentraties aan vetzuren, zoals die bijvoorbeeld voorkomen bij zuurstofgebrek, een schadelijke werking hebben. De aanwezigheid van vetzuurbindende eiwitten is mogelijk van belang bij het tegengaan van dit effect.

De ontwikkelde methoden voor de meting van de vetzuurverbranding in verschillende spierpreparaten en voor de bepaling van vetzuurbindende eiwitten kunnen goed gebruikt worden bij de verdere bestudering van de regulatie van de vetzuur-stofwisseling in de spier.

# STELLINGEN

I

De conclusie van Paul en Adibi, dat toediening van clofibraat aan ratten de vetzuuroxidatie in de skeletspier verstoort, is niet juist.

Paul, H.S. & Adibi, S.A. (1979) *J. Clin. Invest.* 64, 405-412
Dit proefschrift, hoofdstuk 6

II

Bij de interpretatie van hun studie naar het [$^{14}$C]oleaat metabolisme in de geperfundeerde lever van de rat hebben Ide en Sugano ten onrechte de $^{14}CO_2$ productie buiten beschouwing gelaten.

Ide, T. & Sugano, M. (1983) *Biochem. Pharmacol.* 32, 2583-2588

III

De door Osborn en Rogers als tautomere zwitterionen aangeduide structuren van fluoresceine zijn mesomere structuren van hetzelfde zwitterion.

Osborn, R.S. & Rogers, D. (1975) *Acta Cryst.* B 31, 359-364

IV

Het voorkomen van antilichamen in het plasma gericht tegen *Plasmodium* species is niet gecorreleerd aan malaria immuniteit.

Grun, J.L. & Weidanz, W.P. (1983) *Infect. Immun.* 41, 1197-1204

V

Op grond van de analyses van Habib *et al.* en DiBona mag niet geconcludeerd worden dat het collageen gehalte van de glomerulaire basaalmembraan van Alport patiënten lager is dan van die van gezonde personen.

Habib, R., Gubler, M.-C., Hinglais, N., Noël, L.-H., Droz, D., Levy, M., Mahieu, P., Foidart, J.-M., Perrin, D., Bois, E. & Grünfeld, J.-P. (1982) *Kidney Int.* 21, S 20 - S 28

DiBona, G.F. (1983) *J. Lab. Clin. Med.* 101, 817-820

VI

Bij de interpretatie van metingen van deoxyribonucleotide trifosfaat concentraties in de totale cel dient rekening gehouden te worden met het bestaan van subcellulaire pools.

Taheri, M.R., Wickremasinghe, R.G. & Hoffbrand, A.V. (1981) *Biochem. J.* 196, 225-235

VII

De suggestie van Gloster en Harris, dat onder normale omstandigheden myoglobine ook functioneert als vetzuurbindend eiwit, moet worden afgewezen.

Gloster, J. & Harris, P. (1977) *Biochem. Biophys. Res. Commun.* 74, 506-513

Fournier, N.C., Geoffroy, M. & Deshusses, J. (1978) *Biochim. Biophys. Acta* 533, 457-464

Dit proefschrift, hoofdstukken 8 en 11

VIII

Bij de bestudering van de functionele en fysisch-chemische eigenschappen van lipide-bindingseiwitten wordt onvoldoende rekening gehouden met de eventuele aanwezigheid van endogene liganden.

IX

Het woord vriesdrogen is wel, maar het woord droogvriezen is geen correct Nederlands.

X

De verbrandingsgassen van een stadsbus dienen op dakhoogte uitgestoten te worden.

XI

De toenemende file vorming op autosnelwegen geeft aan het rij-bewijs een andere betekenis.

XII

Bij velen wordt de snelheid van schrijven bepaald door de verdampingssnelheid van het oplosmiddel 1,1,1-trichloorethaan.

XIII

Het lijkt tegenwoordig belangrijker om baanscheppend dan om baanbrekend onderzoek te doen.

XIV

Het is twijfelachtig of de term blaastest juridisch waterdicht is.

Nijmegen, 9 december 1983 J.F.C. Glatz