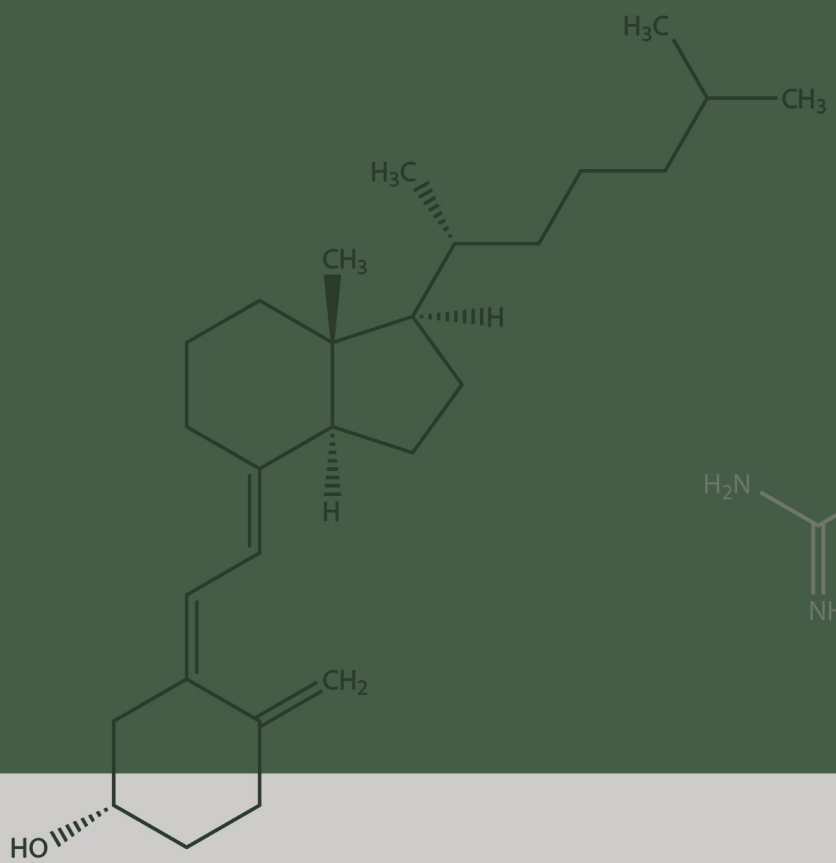
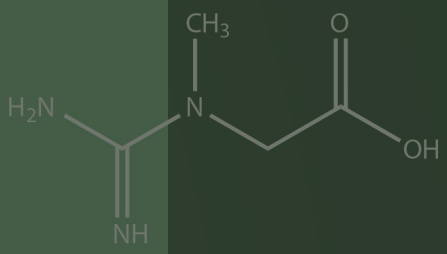
Bond's DIETARY SUPPLEMENTS





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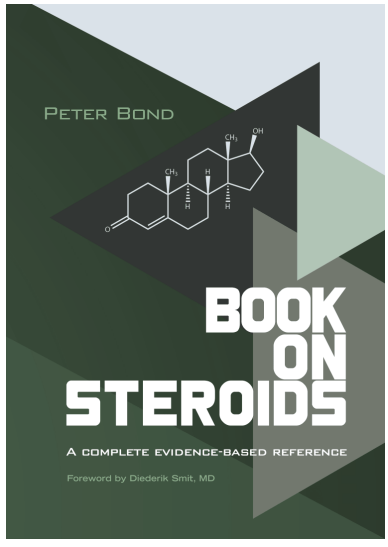
Foreword to the English edition

This book is an English translation of the Dutch book ‘Bonds voedingssupplementen‘ I’ve published earlier in 2017. Many people have been asking me to translate this book to English, so here it is. For free. (If you paid for this book, someone scammed you.) While the book was published in 2017, most of it was written in 2015. As such, it’s unavoidable that some parts of the book might have become outdated by now. Nevertheless, this book will prove to be a valuable resource for anyone looking to get into the depths of the science behind dietary supplements that increase muscle strength or promote muscle growth.

Since I’ve translated this book myself, any spelling or grammar errors are my fault. Please forgive me, as English is not my native language. (And translating something turned out to be quite an undertaking.)

In case you also happen to be interested in the subject of anabolic-androgenic steroids (AAS), take a peek at my brand-new book ‘Book on Steroids’. Chock-full of evidence-based information with well over 500 references to the scientific literature. A must-have on the bookshelf of anyone with a keen interest in AAS. Book on Steroids is available as a paperback, PDF, and Kindle at https://bookonsteroids.com.



If you spot an error in this book, if you have a remark, if I misreferenced something, if I’ve forgotten to reference something, or if you just want to say hi, feel free to drop me an email at peter@peterbond.org.

Peter Bond
Zeist, the Netherlands, November 2020

Foreword

With this book I hope to meet the growing need for evidence-based information about dietary supplements. To this end, the book contains more than 500 inline references to the scientific literature and I have taken care to present the information on the most current state of affairs. The focus of this book is on dietary supplements that increase muscle strength or promote muscle growth. This book also includes various boxes that provide in-depth information to complement the text.

Obviously, not all dietary supplements will be discussed. Otherwise I would have never been able to finish this book. As a consequence, I have made a selection from the range of available dietary supplements. This selection is based on the more popular dietary supplements and the supplements about which sufficient information is available in the scientific literature. Furthermore, the first part of this book covers some fundamental information that will aid in properly interpreting this information and enables you to gather further information from the scientific literature.

Although I have done my best to present the information provided in this book as correctly as possible, it's still possible that errors or inaccuracies have slipped between the cracks and made it into the book. If you encounter such errors or inaccuracies, or if you have any comments or remarks, feel free to drop me an email at peter@peterbond.org.

Finally, I would like to thank my proofreaders, in particular Rob van Berkel, Jorn Trommelen and Peter Van Mol, for their feedback on previous drafts of this book.

Peter Bond
Zeist, the Netherlands, February 2017

Contents

Physiological basis

1. Muscle physiology

1.1 Introduction

Skeletal muscle allows us to move around in our daily lives. Muscles connect the bones of our skeleton with each other. This allows us to move them voluntarily relative to each other. To be clear: whenever I speak about muscle in this book, I'm referring to skeletal muscle and not heart muscle or smooth muscle.

The muscles provide continuous support to our skeleton, so that it doesn't collapse like a card house at rest. About 40–50 % of our body mass consists of muscle tissue [253]. Besides supporting locomotion and structural support for the skeleton, it also plays an important role in the metabolism of our bodies. In athletes, especially bodybuilders, muscle tissue can make up well over half of the entire body mass. Increasing this mass therefore plays a central role in bodybuilding, along with achieving a very low body fat percentage and aesthetic muscle symmetry. The central theme of this book is also focused on dietary supplements that increase muscle mass or promote muscle strength.

To properly understand how dietary supplements work, it's crucial to have an understanding of how our muscles work. That is why this subject is extensively covered in this chapter. This chapter will discuss the macro- and microscopic structure of muscle tissue, the mechanism of muscle contraction, the different types of muscle fiber types, satellite cells, and the neuromuscular control. The macrostructure of muscle tissue views the structure at a 'coarse' level. It answers questions such as: what components does a muscle consist of? And: how are these built up? After all, a muscle consists of more than just the muscle fibers (myocytes) that run from origin to insertion (see Box 1.1).

Box 1.1

The origin and insertion are the two points of attachment of a muscle to the skeleton. The muscle fibers connect to the skeleton via tendon tissue. This way the force that is generated by the muscle fibers can be transferred to bone. Muscle fibers can also be directly attached to bone. Traditionally, the origin is defined as the proximal attachment of the muscle, or the attachment closest to the center of the body. The distal attachment, which is furthest from the center of the body, is called the insertion. It should be noted that muscles can have multiple origins or insertions. For example, the biceps brachii, which can bend the elbow, has two origins (both at the scapula) and one insertion (at the radius).

The muscle fibers form specific structures (bundles) and are supported by surrounding material (connective tissue). When we look at the microstructure of muscle tissue, we look at the tiniest level. A question we'll be answering is: what does a muscle fiber consist of at the molecular level? Then follows an overview of the mechanism of muscle contraction, the primary function of muscles, as described by the sliding-filament model (see Box 1.3). This model elegantly describes how the microscopic structures of a muscle fiber ultimately lead to muscle contraction. We will then also discuss the different muscle fiber types. In it, we explain which microscopic, but especially biochemical, differences there are between the different types. Subsequently, the role of satellite cells is briefly discussed. These cells supply the established muscle cells with new cell nuclei. Finally, it's explained how a voluntary muscle contraction is initiated by a motor neuron, or in other words, the neuromuscular control.

1.2 Macro- and microscopic structure

A muscle consists of muscle tissue, connective tissue, blood vessels and nerve cells. The outer layer of a muscle consists of the fibrous tissue membrane epimysium. The epimysium surrounds the entire muscle and is continuous with the tendons that attach the muscle to bone. Beneath the epimysium we find muscle fiber bundles, which, in turn, are surrounded by the fibrous tissue membrane perimysium. The perimysium connects the muscle bundles to the epimysium. The individual muscle fibers are further surrounded by the endomysium. The connective tissue ensures the transfer of contraction force to the bones, but also protects the individual muscle fibers and allows the muscle fiber bundles to slide past each other during contraction. During a contraction, the shape of a muscle changes significantly.

A muscle fiber is shaped by a plasma membrane, the sarcolemma. In addition to the usual functions that a plasma membrane fulfills, such as the transport of nutrients and waste products, and the integration of external cues, it also conducts the action potential of the adjacent motor neuron to initiate contraction (see Box 1.2).

Box 1.2

The plasma membrane separates the inside of a cell (intracellular) and the outside of a cell (extracellular). This separation is established by phospholipids that comprise a bilayer. The plasma membrane also contains proteins that allow (selective) transport of certain molecules. For example, there are proteins that form channels for ions such as Ca^{2+}, but also for large molecules such as glucose. Lipid vesicles from the outside can also 'fuse' with the membrane in order to release its contents into the cell (endocytosis). This

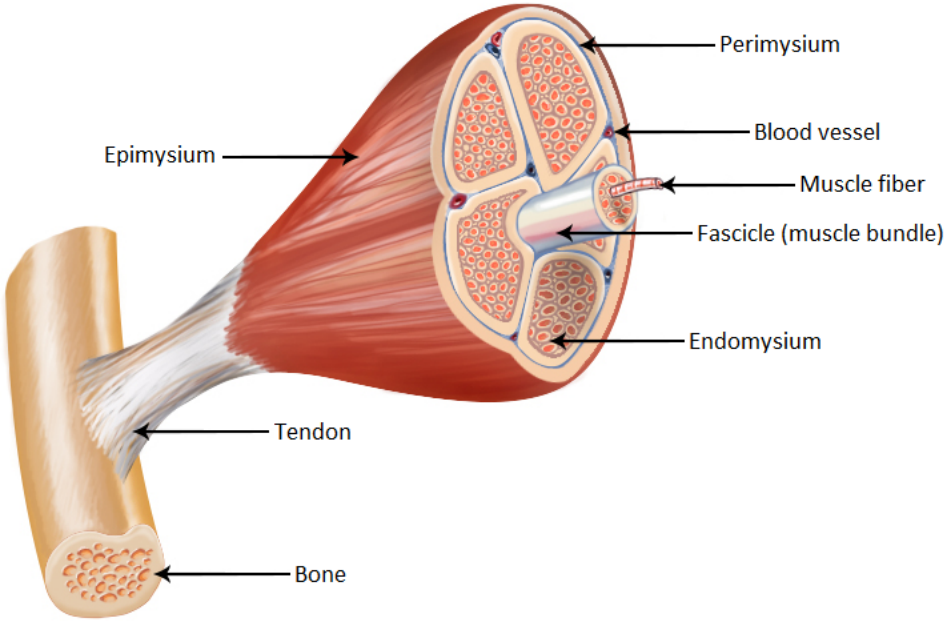


Figure 1.1: The macroscopic structure of skeletal muscle tissue. Image: National Institutes of Health.

way material from the outside ends up intracellularly. Conversely, the cell can also make vesicles filled with substances to release some of its contents (exocytosis). Additionally, the cell is equipped with channels and transport proteins to transport substances to the outside.

Besides the exchange of substances, a cell can pick up extracellular signals. The plasma membrane contains receptors to receive signals from the outside, such as the insulin receptor. Binding of the peptide hormone insulin to the insulin receptor on the sarcolemma activates a pathway that causes more glucose transporters to be present on the sarcolemma. This way insulin can stimulate the absorption of glucose by muscle tissue. In addition, muscle tissue has the unique ability to translocate (move) more glucose transporters to the sarcolemma independently of insulin action in response to contraction. Particularly important for muscle cells is the regulation of ion transport along the sarcolemma, which allows an action potential to be propagated across the sarcolemma. This process is further explained in Section 1.6.

Within the sarcolemma is the sarcoplasm (the cytoplasm of a muscle fiber), consisting of the cytosol (a gelatinous liquid) and the organelles. Organelles are components of the cell that are also surrounded by a membrane and perform unique functions. For example, the mitochondria generate the energy to allow cellular processes to take place, and within the cell nucleus gene transcription occurs.

The sarcoplasmic reticulum (SR, the endoplasmic reticulum of a muscle fiber) fulfills a unique role in muscle fibers. The sarcolemma has extensions that span into the centre of the cell and are closely associated with the SR. These extensions are called the transverse tubules (T-tubules). This enables the T-tubules to transmit the start signal for contraction

to the SR. The SR acts as a Ca^{2+} storage site. Once the signal for contraction is received by the SR, these calcium ions are released into the cytosol. The SR forms a network of tubes through the muscle fiber that surrounds the myofibrils, along with the T-tubules.

The myofibrils are the contractile proteins of the cell. They consist of different types of proteins that enable muscle contraction. The myofibrils look like wires that run the length of the fiber. A lot of sarcomeres in series form a single myofibril.

A sarcomere is the smallest functional unit of a muscle fiber that can contract. The proteins actin and myosin play a central role in this. These proteins 'slide' over each other and thus cause contraction. The proteins troponin and tropomyosin together regulate when a sarcomere contracts. The calcium ions from the SR are responsible for a conformational (spatial) change in the structure of these proteins that initiates contraction.

Actin, tropomyosin and troponin make up the thin filament. Actin–by far–makes up the largest part of the thin filament. The protein is spherical (globular actin [G-actin]), but as a polymer it forms a long double helix (fibrillar actin [F-actin]). The backbone of F-actin is formed by the protein tropomyosin, along which G-actin is bound.

A troponin-protein complex can be found after every seven G-actin molecules. The troponin-protein complex consists of three subunits, namely troponin I, troponin T, and troponin C. Troponin I ensures binding to actin and troponin T ensures binding to tropomyosin. Troponin C has a high affinity for Ca^{2+} and causes a conformational change of the complex upon binding thereto. This conformational change means that the actin is no longer inhibited by the complex to bind to the myosin heads. The myosin protein forms the thick filament, which is bonded to the Z-disks with titin on both sides.

Myosin, in turn, consists of as many as six subunits. Two of these subunits are so-called heavy chains (MHC, myosin heavy chain) and four are light chains (MLC, myosin light chain). The MHC also take on a double helix shape, with structures that resemble heads on either side. The MLC mainly play a supporting and regulatory role for these heads. The myosin heads are ultimately responsible for contraction: they 'walk', as it were, over the actin and thus pull it towards themselves. The mechanism of muscle contraction is discussed further in Section 1.3.

1.3 Muscle contraction

The contraction of a muscle is initiated by a depolarization of the sarcolemma. The action potential responsible for this depolarization is triggered by a motor neuron. This is further described in Section 1.6. L-type Ca^{2+} channels that are linked to adjacent Ca^{2+} channels of the SR open as soon as the action potential is passed along the T-tubules. This leads to a calcium-induced outflow of Ca^{2+} from the SR into the cytosol. This causes the cytosolic concentration of Ca^{2+} to rise sharply. As a result, more Ca^{2+} binds to troponin C, as described in section 1.2, and thus an association between actin and the myosin heads can arise: the acto-myosin-complex. After formation of this complex, ATPases on the myosin heads can hydrolyze the energy-carrying molecule adenosine triphosphate (ATP) to release adenosine diphosphate (ADP) and an inorganic phosphate group (P_i). These two products associate with myosin, and the free energy released from the hydrolysis of ATP leads to a rotation of myosin, forming a cross-bridge with actin. The rotation of myosin puts a strain on the protein. Much like a compressed spring, the protein in this state holds energy. This energy causes a kink in the neck of the myosin head when the P_i group dissociates from the myosin. This kink 'pulls' the thin filament over the thick filament, towards the cneter

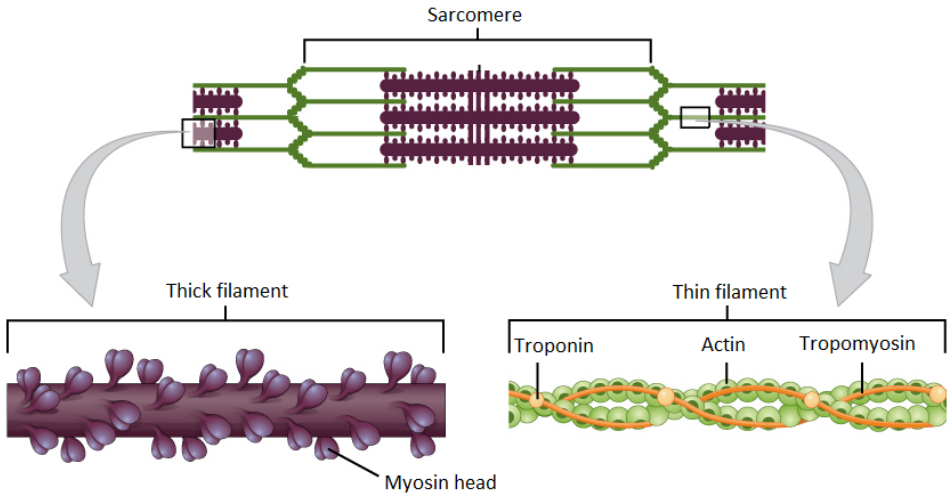


Figure 1.2: A sarcomere. The thick filament consists of myosin proteins and the thin filament consists of troponin, actin and tropomyosin. The Z-disks are located on either side of the sarcomere. The thick filaments are bound to this with titin. Image taken and adapated from OpenStax Anatomy and Physiology. Distributed under the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/deed.nl).

of the sarcomere. This is also called the power stroke. After this, the still bound ADP dissociates and the cycle can repeat itself as long as ATP is present.

Box 1.3

The sliding filament model was introduced independently and simultaneously by two research groups (Andrew Huxley and Rolf Niedergerke, University of Cambridge and Hugh Huxley and Jean Hanson, Massachusetts Institute of Technology). Both groups published their findings in Nature in May 1954. It was no coincidence that their work was published simultaneously, as both groups were aware of each others work [231]. The model has remained essentially unchanged since its introduction.

1.4 Muscle fiber types

A muscle consists of different types of muscle fibers. The subdivision of these types can be made on various grounds. Long ago, a distinction was already made between white muscle tissue and red muscle tissue. Clearly, a distinction was made on the basis of their phenotype.

The color of red muscle tissue can be attributed to the high concentration of myoglobin it contains. Myoglobin has a heme group which is responsible for the red color. Heme groups can also be found in the red blood cell protein hemoglobin and is thus also the reason for blood to be colored red. Myoglobin is responsible for intracellular oxygen transport and thus the metabolism of red muscle tissue indeed heavily relies on oxygen.

Besides the distinction that can be made on phenotype, muscle fibers can be distinguished on the basis of their contractile properties. For example, fast and slow muscle fibers can be distinguished by looking at how long it takes to reach maximum force after muscle fiber contraction is initiated. Note that the designations of 'fast' and 'slow' are mainly important for characterization and not so much for practical purposes in daily life: you don't notice.

A distinction can also be made on the basis of metabolic properties; aerobic or anaerobic. An aerobic (oxidative) muscle fiber has many mitochondria, is highly vascularized and has a high myoglobin and oxidative enzyme content. All these features are designed to ensure the muscle fiber is optimally supplied with oxygen and to put this to use to generate energy. On the other hand, an anaerobic muscle fiber has few mitochondria, is less vascularized, has a low myoglobin content and is characterized by a high glycolytic capacity (an anaerobic way of generating energy which is discussed in Section 2.3). However, it should always be kept in mind that this mainly indicates extremes of the muscle fiber types. In reality, many muscle fibers tend to fall between between these two types of muscle fiber classification.

With the help of immunohistochemistry and elektrophoresis, the popular subdivision based on the major MHC isoform present in a muscle fiber arose. Based on the expression of the MHC isoforms one can distinguish four primary muscle fiber types, namely: type 1 (MHC1β), type 2A (MHC2a), type 2X (MHC2x), and type 2B (MHC2b) [405]. Type 2B is not present in humans in any of the major skeletal muscles. The muscle fibers that express the type 1 isoform are also called slow twitch fibers and the muscle fibers that express the type 2 isoforms are called fast twitch fibers. The MHC isoform contributes to the properties of the different types and can be accurately identified, which is why this classification is often seen as the leading one. The speed with which a muscle fiber contracts is correlated with the respective MHC isoform [352]. The sequence of contraction speed from fast to slow is as follows:

type 2B > type 2X > type 2A > type 1

An important factor here is the speed with which the myosin heads of the different MHC isoforms can hydrolyze ATP to make the power stroke and thus slide the filaments over each other.

As emphasized earlier, many muscle fibers' classification fall between these types, these are also called hybrid muscle fibers. They express two different MHC isoforms and to varying degrees.

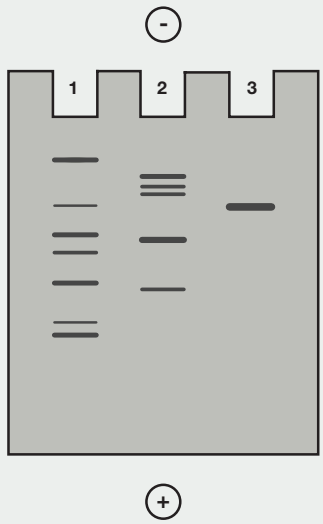
Also, so-called transition muscle fibers have been hypothesized, which may contain an as yet unknown MHC isoform, have certain post-translational modifications or express an aberrant isoform of other myofibrillar proteins [326].

Most fibers are likely to be 'pure', i.e., expressing only one MHC isoform. This is probably due to the make-up of the type 1 isoform gene. This gene contains two introns that each describe a miRNA (miR-208b and miR-499). These miRNAs block the translation of transcription factor Sox6 [458, 368]. This transcription factor is responsible for inhibiting expression of the type 1 isoform whereas it stimulates the expression of the fast isoforms. As such, these two miRNAs function much like an on/off switch—determining which MHC isoform is expressed.

Box 1.4

Immunohistochemistry is a technique that can be employed to detect certain proteins. It relies on a molecule (antibody) that is specific for the protein to be detected (antigen). An antibody can bind to its antigen and thus form an antibody-antigen complex. Once such a complex is formed, it must be separated from the rest of the molecules. After this separation follows the detection and quantification of the complexes. This can be done in several ways. Popular methods for this are a western blot, a radioimmunoassay (RIA) or by coupling to an enzyme that catalyzes a color reaction (enzyme-linked immunosorbent assay [ELISA]).

Electrophoresis can separate proteins based on their size or charge. A gel is used with a negative electrode on one side and a positive electrode on the other. This creates an electric field from one electrode to the other.
Using polyacrylamide gel elektrophoresis (PAGE), in which pre-treatment with sodium dodecyl sulphate (SDS) is required first, separation is based on size (SDS-PAGE). When the protein mixture is released into the gel on the side of the negative electrode, the individual proteins will migrate to the positive electrode at different rates. The speed at which they migrate depends on the size of the protein, and thus separation can be based on size.



The figure above shows an example of the separation of proteins using SDS-PAGE. To visualize the proteins (the lines in the figure), autoradiography, direct staining with dyes or indirect staining with antibodies can be utilized. In the latter case, the separated proteins are first transferred to a membrane to which they adhere (blotting).

In order to determine the sizes of the proteins, a reference column is needed. To this end, a protein mixture of which the masses of the different proteins are known is used (column 1 of the figure). The mixtures in columns 2 and 3 can thus be compared to this reference column. The lines closer to the negative electrode have a larger molecular mass than the lines closer to the positive electrode.
With isoelectric focusing (IEF) separation is based on the charge of proteins rather than their size. After all, proteins consist of numerous amino acids and amino acids contain side chains that have an electrical charge. A gel with a pH gradient is used with this technique, which causes the proteins to stop across the pH gradient of the gel at the point in which the overall charge of the protein is 0 (the isoelectric point).

It's important to realize that a muscle fiber's classification certainly doesn't fully dictate the specific properties of that fiber. However, it provides some guidance that, in general, is convenient in use.

1.5 Satellite cells

Muscle fibers contain multiple nuclei and for that reason they are also called multinucleated cells. Because the cell nuclei of muscle fibers are unable to divide (post-mitotic), they rely on an external source for replenishment and regeneration of cell nuclei. Rat muscle fibers contain about 44 to 116 nuclei per millimeter, with type 1 muscle fibers containing more nuclei per millimeter than type 2 muscle fibers [450]. Since muscle fibers can reach several centimeters in length, this means that a single muscle fiber can already contain hundreds to thousands of cell nuclei.

These cell nuclei mainly stem from the surrounding satellite cells. It's estimated that, at a given moment, 1.4 % to 7.3 % of all nuclei in a muscle are contained in satellite cells [248]. Satellite cells are located close against the muscle fibers, between the sarcolemma and the basal lamina. They were first discovered by Alexander Mauro [301]. With an electron microscope aimed on muscle fibers of the tibialis anterior of a frog, he saw cells 'wedged' between the plasma membrane of the muscle fiber and the basal lamina. He also noted that these satellite cells contained almost no cytoplasm and the nucleus made up almost the entire volume of the cells. Several hypotheses were briefly discussed by him, including that they could serve to repair damaged muscle fibers. Later research, indeed, demonstrated this to be the case [103, 391].

Not much later, the role of satellite cells in muscle hypertrophy was explored. An important question arose: do muscles grow because the mRNA and protein synthesis per cell nucleus increases, or does it remain the same and are more cell nuclei added to the muscle fiber? In the latter case, satellite cells would fulfill an extremely important role. Some findings eventually led to the so-called myonuclear domain hypothesis. This hypothesis posits that each cell nucleus can only control a limited amount of cytoplasm, so for muscle growth to occur, new cell nuclei must be added to the muscle fiber in order to support this growth. This hypothesis mainly stemmed from three findings, namely that:

- γ-radiation, which effectively destroys almost all satellite cells, strongly inhibited overload hypertorphy [389];
- proteins synthesized by a cell nucleus confine themselves to a specific region (the myonuclear domain) [345]; and
- the amount of cytoplasm around a cell nucleus (the ratio cytoplasm/cell nucleus) remains fairly constant [11].

However, several experimental models were later able to demonstrate (optimal) hypertrophy without the need for satellite cells [49]. These data made it reasonable to state that satellite cells therefore aren't required for muscle hypertrophy. One study even showed a $\sim$50 % increase in muscle mass in a transgenic mouse model with a constitutively active form of Akt (Akt is an important protein for muscle growth, among other things, and is discussed in Section 4.2.1) [48].

Nevertheless, it should be noted that all of these experiments only looked at the short-term (several weeks) results. Moreover, recent research sheds a different light on these results [141]. For example, these earlier findings, in which satellite cells appear not to be required, relied on measured mass as a surrogate endpoint for muscle hypertrophy.

This can produce unreliable results because it's difficult to anatomically define some of these muscles (especially the plantaris muscle) in the used animal models. Damaged or regenerating muscle fibers would also incorrectly be included.

The number of satellite cells is usually determined with the use of immunohistochemistry. The transcription factor Pax7 is commonly used as a biomarker for this. When satellite cells are subsequently activated, they form myoblasts and proliferate. These activated satellite cells are characterized by the expression of Myf5 and MyoD. These cells can then further differentiate into myotubes, which are characterized by the expression of myogenin and Myf6. Finally, the myotubes can fuse with muscle fibers in order to donate their nuclei.

The regulation of this proliferation, differentiation and fusion in the context of muscle growth is regulated by the muscle fibers. This is a form of paracrine regulation: the muscle fibers produce molecules that activate the surrounding satellite cells. This regulation is chiefly controlled by serum response factor (Srf), a transcription factor that is regulated by mechanical load and results in the secretion of the interleukines IL-6 and IL-4 by the muscle fiber. IL-6 and IL-4, in turn, take care of the proliferation and fusion of the satellite cells with the muscle fibers, respectively [187].

1.6 Neuromuscular control

Muscles are controlled by motor neurons (see Box 1.5). These neurons originate in the spinal cord and project to the muscles, where they commonly split into many nerve twigs. Each twig controls a single muscle fiber. That way a motor neuron innervates several muscle fibers. The number of muscle fibers that a motor neuron innervates can vary from just a few to even hundreds of muscle fibers. The entirety of a motor neuron and the muscle fibers innervated by it is referred to as a motor unit. The motor neuron triggers its innervated muscle fibers by means of a neuromuscular junction (also called motor endplate). The neuromuscular junction is where the motor neuron makes contact with the muscle fiber. The end of the neuron (the axon terminal) is located at a very close distance (the synaptic cleft) from the sarcolemma. These axon terminals are characterized by the synaptic vesicles they contain.

Box 1.5

A motor neuron is a specialized type of nerve cell (neuron). Neurons have a general structure consisting of a signal-receiving part (dendrites), a cell body (soma), an elongated conducting part (axon) and finally a transmission hatch of the signal (axon terminal). The space between the axon terminal and the cell that receives the signal (postsynaptic cell) is called the synaptic cleft. Once an action potential is transferred to the soma and is carried along the axon and reaches the axon terminal, the neuron will secrete a chemical substance in the synaptic cleft. This chemical is a neurotransmitter. The body has many neurotransmitters, such as the catecholamine noradrenaline or the ester acetylcholine. The neurotransmitter then binds to receptors located on the postsynaptic cell, where it triggers a response. Acetylcholine is stored in the synaptic vesicles and is secreted by the axon terminal of a motor neuron once triggered. After this, it binds to acetylcholine receptors located on the sarcolemma, triggering the depolarization of the muscle fiber.

A motor neuron stores the transmitter acetylcholine in its synaptic vesicles. The acetylcholine is secreted into the synaptic cleft by exocytosis when the motor neuron is

triggered. The acetylcholine binds to receptors present on the postsynaptic membrane (the sarcolemma). This makes the membrane more permeable to cations. This increased cation permability results in membrane depolarization. The acetylcholine that is released into the synaptic cleft is quickly cleared again in order to prevent the continuous stimulation of its associated muscle fiber. This is done by acetylcholinesterases present on the sarcolemma.

A single contraction of a muscle fiber is called a twitch. In reality, twitches are rare. Motor neurons are often triggered several times for even the most delicate and short-lasting movements. By increasing the frequency with which the motor neuron depolarizes and secretes acetylcholine in the synaptic cleft, and as a result excites the muscle fiber, the motor neurons are able to realize a continuous contraction (and maximum contraction force). At a sufficiently high frequency, the concentration of Ca^{2+} in the muscle fiber rises to such a high level that the maximum amount of cross bridges is formed. This is called a smooth tetanus. In smooth tetanus, the depolarizations follow each other so quickly that no relaxation (Ca^{2+} reuptake by the SR) of the muscle fiber occurs. The Ca^{2+} concentration therefore remains high enough to bind almost all the troponin C. This exposes the thin filaments and allows the maximum number of cross bridges to be formed with the thick filaments. When there is sufficient time between the successive depolarizations for partial relaxation, a rough tetanus develops (see Figure 1.3).

The whole process, starting with the signal from the brain that causes the innervated muscle cells to contract through a motor neuron, is called excitation-contraction coupling.

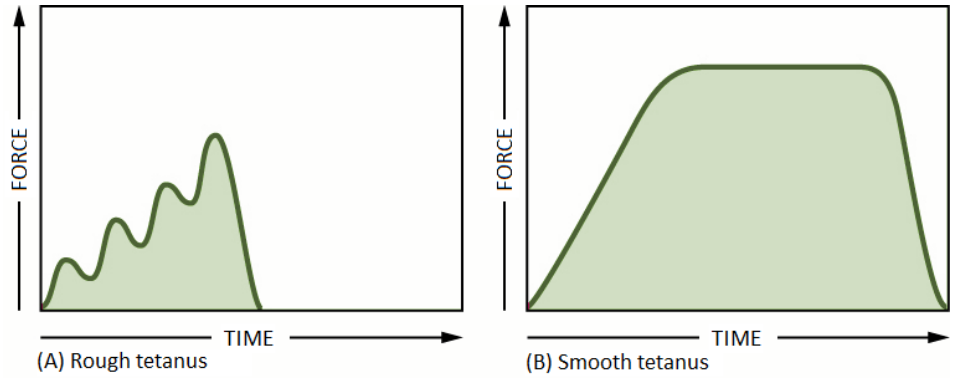


Figure 1.3: (A) The successive depolarizations lead to a summation of the force delivered, but the intervals are still long enough to allow relaxation. The individual depolarizations are still clearly visible with a rough tetanus (also called an incomplete tetanus). **(B)** The depolarizations follow each other so quickly that relaxation no longer occurs and the individual depolarizations are no longer visible. Smooth tetanus is occurring. Figure taken and adapted from OpenStax Anatomy and Physiology. Distributed under the Creative Commons Attribution 4.0 International license (https://creativecommons.org/licenses/by/4.0/deed.nl).

2. Energy systems

2.1 Introduction

Muscles need energy in order to contract. The muscle fibers, like all other cells, obtain this energy from glucose and fatty acids. Muscle fibers are special cells when it comes to extracting energy from fuels. Whereas most cells have a fairly constant rate of energy expenditure, energy expenditure of a muscle fiber can increase more than a hundredfold from one moment to the next [393]. Fortunately, muscle fibers are equipped with three very well-developed energy systems. These three energy systems differ in their capacity (the amount of work that can be done) to provide energy and the rate at which they can do this (power).

For example, the phosphagen energy system has a very small capacity, just enough to maintain a muscle contraction for a few seconds [472]. However, it can release this energy rapidly [393], i.e. it has high power. It also doesn't consume oxygen. The phosphagen energy system therefore plays an important role in intense, very short, bouts of exercise.

The glycolytic energy system has a somewhat larger capacity than the phosphagen energy system, but its power is lower. This system can therefore supply energy for a longer period of time, but produces less energy per unit time than the phosphagen energy system. Just like the phosphagen energy system, this system works anaerobically and therefore doesn't consume oxygen.

The oxidative energy system has even lower power, but has—practically speaking—an endless capacity. As the name suggests, this system does consume oxygen (aerobic).

It's important to realize that both the glycolytic energy system and the oxidative energy system extract their energy directly from the fuels we consume in our diet: carbohydrates, fatty acids and amino acids. The phosphagen energy system, however, relies on these

two energy systems to replenish itself, which happens during rest. All things considered, the phosphagen energy system therefore functions as an intermediate buffer of rapidly available energy.

Finally, these systems shouldn't be seen as sequentially operating suppliers of energy, but as continuously operating and cooperating systems. Even with a maximum effort exertion of just a few seconds, some of the energy, although small, is derived aerobically. The longer the effort, the greater the contribution of the oxidative energy system will be to meet the energy demand. It's estimated that at maximum effort exertion an equal contribution of anaerobic and aerobic energy occurs between 1 to 2 minutes, and probably around 75 seconds [159]. And although traditionally maximum efforts of 30 seconds are seen as purely anaerobic, here also more than a quarter of the energy seems to be aerobically generated.

	Capacity (J/kg)	**Power (W/kg)**
Phosphagen energy system	400	800
Glycolytic energy system	1000	325
Oxidative energy system	-	200

Table 2.1: The capacity and power of the three energy systems expressed in joules per kg of muscle mass. These values are estimates only and are highly dependent on training status and other factors. Note: 1 W = 1 J/s; power thus expresses a measure of energy per unit of time. Taken from Cardinale et al. [83].

2.2 Phosphagen system

The phosphagen energy system yields energy in a form that can be directly utilized by many cellular processes, including the contraction of the sarcomeres. This form of energy is the molecule adenosine triphosphate (ATP), which, among other things, is used by the myosin heads to perform the power stroke as described in Section 1.3. When contracting, ATP is used very rapidly and is also rapidly replenished by this energy system. As a result, the concentration of ATP in the cell does not decrease much. The ATP turnover is very large. In order to run a marathon, the body of an elite marathon runner regenerates as much as 60 kg ATP [73].

The reaction that the myosin ATPases catalyze to extract energy from an ATP molecule is as follows:

$$\mathrm{ATP}^{4-} + \mathrm{H_2O} \rightarrow \mathrm{ADP}^{3-} + \mathrm{P_i}^{2-} + \mathrm{H}^{+}. \tag{2.1}$$

The ATP is hydrolyzed and this yields adenosine diphosphate (ADP), inorganic phosphate ($\mathrm{P_i}$) and a proton (H^{+}). However, the amount of ATP in a cell is very limited. The ATP concentration in a muscle fiber is 2–5 mM, this is just enough to sustain muscle contraction for a few seconds [472].

The phosphagen energy system consists of a reservoir of creatine (Cr), creatine phosphate (PCr) and the enzyme creatine kinase (CK). CK catalyzes the following reversible reaction:

$$\mathrm{PCr}^{2-} + \mathrm{ADP}^{3-} + \mathrm{H}^{+} \leftrightarrow \mathrm{Cr} + \mathrm{ATP}^{4-}. \tag{2.2}$$

With this knowledge we can deduce what happens on an energy level when a muscle contracts. The muscle fiber receives the signal to contract causing the myosin ATPases on the myosin heads to hydrolyze ATP (Reaction 2.1). This yields ADP and protons (H^+), as can also be seen on the left side of Reaction 2.2. The PCr buffer of the phosphagen energy system can now steer Reaction 2.2 to the right, so that ATP is immediately regenerated again. Moreover, the generated protons will be directly consumed as well, so that this reaction kind of functions as a pH buffer if you will. A drop in pH resulting from the reaction catalyzed by the myosin ATPases is thus prevented.

The PCr buffer can be completely replenished again at rest. This happens with contributions from the glycolytic energy system and the oxidative energy system once there is enough ATP to drive Reaction 2.2 to the left. This happens in both the cytosol and the mitochondria. In the mitochondria, the oxidative energy system generates ATP. As a result, a lot of ATP is present in the mitochondria during muscle contraction compared to the myofibrils where the ATP is consumed. This causes the CK-catalyzed reaction to move to the right in the cytosol to generate ATP, whereas in the mitochondria it moves to the left to yield PCr. The PCr generated in the mitochondria then diffuses to the myofibrils where it's used to generate ATP again. This is also called the creatine phosphate shuttle system (see Figure 2.2.1).

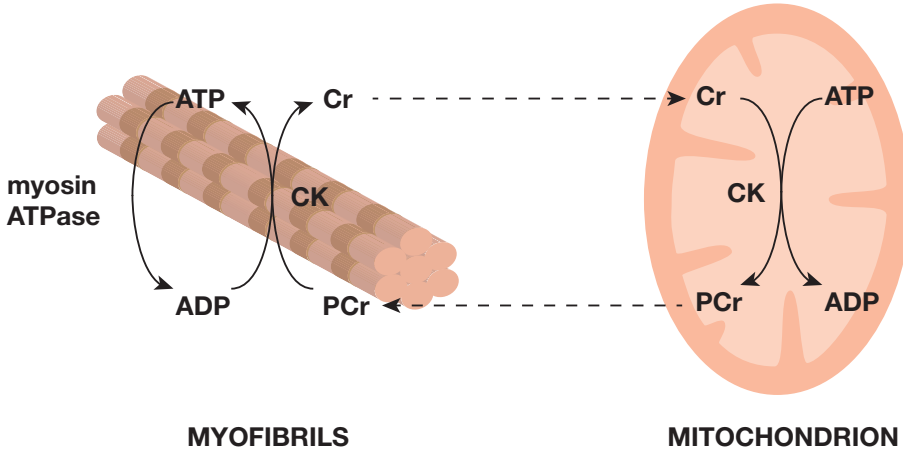


Figure 2.1: The creatine phosphate shuttle system. The myosin ATPases hydrolyze ATP in order to contract, which leads to the generation of ADP. The CK-catalyzed reaction, in turn, can then rephosphorylate the ADP to ATP using the phosphate group from PCr. The resulting Cr diffuses back to the mitochondria where it's phosphorylated by CK back to PCr, using energy derived from ATP generation by the oxidative energy system. Abbreviations: ADP, adenosine phosphate; ATP, adenosine triphosphate; CK, creatine kinase; Cr, creatine; PCr, creatine phosphate.

Box 2.1

i Creatine supplementation can increase the total amount of Cr (and thus also

the amount of PCr) in muscle fibers. This is the basis for the performance-enhancing effect of creatine supplementation. Creatine supplementation is discussed further in Chapter 8.

2.3 Glycolytic energy system

The glycolytic energy system is based on the energy released by the biochemical process called glycolysis. Glycolysis splits a molecule of glucose into two molecules of pyruvate. This generates two molecules of ATP, as well as two molecules of NADH. The pyruvate can then be converted to acetyl-CoA to serve as a substrate for the citric acid cycle, as described in Section 2.4.1. However, when there is a lack of oxygen, the pyruvate is mainly reduced to lactate. This is a reaction catalyzed by lactate dehydrogenase (LDH). Lactate is also produced at rest, but in smaller quantities.

Glycolysis consists of ten steps that are catalyzed by enzymes. In summary, these ten steps perform the following reaction:

$$\begin{aligned} \text{glucose} + 2\ \text{NAD}^+ + 2\ \text{ADP} + 2\ \text{P}_\text{i} \rightarrow \\ 2\ \text{pyruvate} + 2\ \text{NADH} + 2\ \text{ATP} + 2\ \text{H}_2\text{O} + 2\ \text{H}^+. \end{aligned} \tag{2.3}$$

As seen in the equation above, the energy generated is limited. Only two ATP molecules are produced. It should be noted that the formed NADH can also—granted there is sufficient oxygen—yield 1.5 ATP per molecule via the glycerol phosphate shuttle [40].

The glucose that forms the substrate for glycolysis either stems directly from the circulation or is supplied by the glycogen storage of the muscle fiber in the form of glucose 6-phosphate. In the latter case, glycolysis yields three instead of two molecules of ATP, which will be covered later. Muscle fibers can take up glucose by facilitated diffusion (transport mechanisms are discussed in Section 3.2). To this end, muscle fibers express glucose transporters, namely GLUT1 and GLUT4.

GLUT1 can be viewed as a transporter responsible for a basal influx of glucose for the muscle fibers and is only expressed to a small degree. GLUT4, on the other hand, takes on the primary role for glucose influx in muscle fibers and is regulated by both insulin and muscle contraction [289]. Without a stimulus (insulin or muscle contraction), GLUT4 is almost completely absent from the sarcolemma and is located intracellularly in lipid vesicles [70]. Because the GLUT4 is stored in these vesicles, the cell can swiftly respond to a stimulus to absorb glucose. Insulin binding to the insulin receptor activates a pathway that leads to rapid translocation of the intracellular GLUT4 to the sarcolemma and the T-tubules.

How muscle contraction leads to GLUT4 translaction to the sarcolemma and T-tubules is poorly understood. The sharp rise in Ca^{2+} might play a role, but also the actin cytoskeleton, nitric oxide synthase activity and the energy sensor AMP-activated protein kinase (AMPK) [383]. It has also been hypothesized that there are two 'different' types of GLUT4 storage sites. One responding to binding of insulin to the insulin receptor and the other responding to muscle contraction. This may explain why there is an additive effect of insulin and muscle contraction on glucose uptake by muscle tissue.

Once the glucose is taken up by the muscle fiber, it's phosphorylated to glucose 6-phosphate by a hexokinase. This ensures that a concentration difference between the

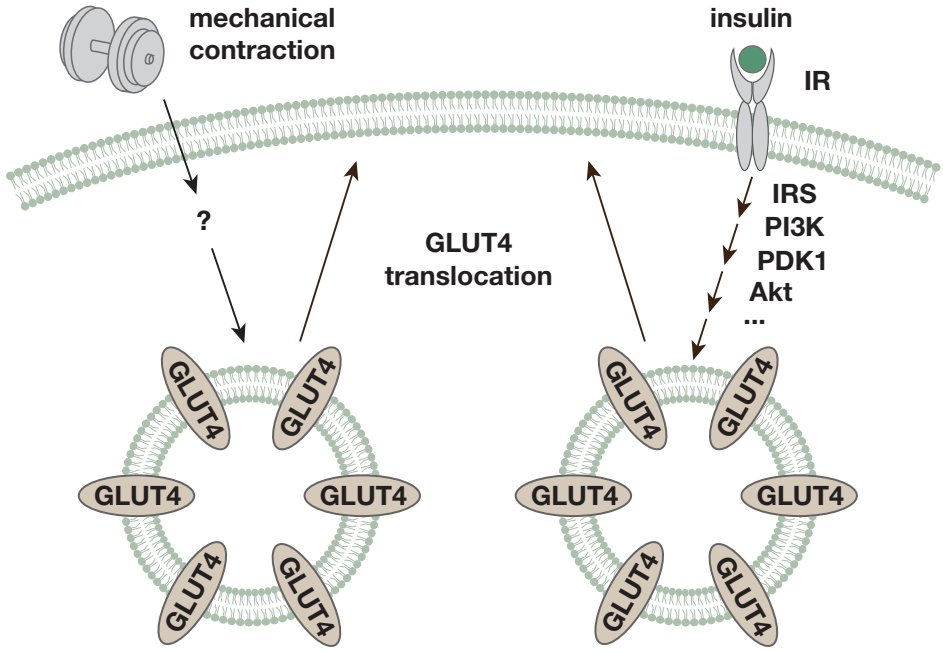


Figure 2.2: GLUT4 translocation in response to mechanical contraction and insulin. Both mechanical contraction and activation of the IR lead to translocation of GLUT4 to the sarcolemma. It has been hypothesized that mechanical contraction and IR activation act on different GLUT4 stores. Abbreviations: GLUT4, glucose transporter 4; insulin receptor, IR; IRS, insulin receptor substrate; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase.

inside of the muscle fiber and the interstitial fluid is maintained. This is necessary as the glucose transporters only facilitate the diffusion along the sarcolemma: there is no active transport against a concentration gradient taking place. Once phosphorylated, the glucose 6-phosphate is 'locked up' in the cell. Muscle fibers do not express the enzyme (glucose 6-phosphatase) to dephosphorylate the molecule. The liver does express this enzyme and thus can convert stored glucose (in the form of glycogen) back into glucose and release it into the circulation to supply other tissues with it.

The formation of glucose 6-phosphate costs one molecule of ATP. This explains the aforementioned difference between the glycolysis ATP yield of glucose versus glycogen. No ATP is consumed to create glucose-6-phosphate from the stored glycogen. Subsequently, the glucose 6-phosphate is converted into fructose 6-phosphate, which is then converted into fructose 1,6-biphosphate. This costs another ATP molecule. These steps are therefore also referred to as the 'investment phase' of glycolysis: they cost energy while not yielding anything yet.

After the fructose 1,6-biphosphate is formed, it's split into the two triose phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The latter is then also converted into glyceraldehyde 3-phosphate. Subsequent steps then form pyruvate from

glyceraldehyde 3-phosphate, yielding two molecules of ATP for each molecule (so four in total). This is referred to as the 'recovery phase' or 'energy payoff phase'. In summary, thus, two molecules of ATP are consumed and four are generated, resulting in a net yield of two molecules of ATP per molecule of glucose.

Besides glucose, the monosaccharides fructose and galactose are also utilized to yield energy through glycolysis. Fructose, present in fruit and part of table sugar (sucrose), is phosphorylated by the enzyme fructokinase to fructose 1-phosphate. Subsequently, it's cleaved by the enzyme aldolase resulting in dihydroxyacetone phosphate (as is the case with fructose 1,6-biphosphate) and glyceraldehyde. The latter is then phosphorylated to glyceraldehyde 3-phosphate and thus also feeds into the glycolysis.

Galactose, part of the disaccharide lactose found in dairy products, is also first phosphorylated on its first carbon atom to form galactose 1-phosphate. This reaction is catalyzed by galactokinase. The enzyme galactose 1-phosphate uridylyltransferase then forms, together with UDP-glucose, glucose 1-phosphate and UDP-galactose. Subsequently, an epimerase converts the UDP-galactose into UDP-glucose and phosphoglucomutase converts the glucose 1-phosphate into glucose 6-phosphate, which then undergoes the same reactions as previously described (the production of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate).

2.3.1 Cori cycle

The end product of glycolysis, pyruvate, has two metabolic destinations. If sufficient oxygen is available (aerobic conditions), the pyruvate is transported to the mitochondria, where it can be further oxidized to yield energy. However, if there's insufficient oxygen available (anaerobic conditions), pyruvate will be reduced to lactate or transaminated to alanine. Although lactate is continuously produced, its production increases significantly during intensive exercise.

The enzyme lactate dehydrogenase (LDH) is responsible for the conversion of pyruvate to lactate, a reaction coupled to the oxidation of NADH to NAD^+. The reaction is reversible and does not yield energy. It does, however, ensure that sufficient NAD^+ remains present to maintain glycolysis flux (see the left side of Reaction 2.3). Additionally, a proton is consumed. As such, the production of lactate from pyruvate diminishes the rise in proton concentration caused by ATP hydrolysis and glycolysis [384]. Lactate can be converted back into pyruvate again, or be transported by the blood circulation to the liver. In the liver, lactate can be converted back into glucose, which can then be transported back to muscle tissue again. This cycle is called the Cori cycle (see Figure 2.3).

Also, when there's a low glycolytic flux, lactate can be oxidized in the mitochondria. This oxidation mainly takes place in the oxidative type 1 muscle fibers, while the production of lactate mainly takes place in the glycolytic type 2 muscle fibers [166, 67]. Therefore, a lactate exchange seems to take place between both muscle fiber types. This intercellular exchange of lactate is part of what is called the lactate shuttle hypothesis.

Analogous to the Cori cycle, there is a so-called glucose-alanine cycle. Muscle cells are able to oxidize branched-chain amino acids (BCAAs). In order to do this, transamination of the amino acid first needs to take place. The amino group is transferred to α-ketoglutarate. As a result, α-ketoglutarate turns into glutamate, and the amino acid in question is changed into its α-keto acid, which can be oxidized. After this, another transamination reaction takes place. The amino group that just got added to form glutamate will be transferred to pyruvate. This yields alanine (and the glutamate is converted back into α-ketoglutarate

again). The formed alanine then leaves the muscle cell, after which it will reach the liver. Here, it will be transaminated with α-ketoglutarate to form pyruvate. The pyruvate is converted into glucose which is then released into the circulation again—making it available for muscle tissue. The formed glutamate can also be converted into glutamine, but its production does not play a significant role in energy metabolism.

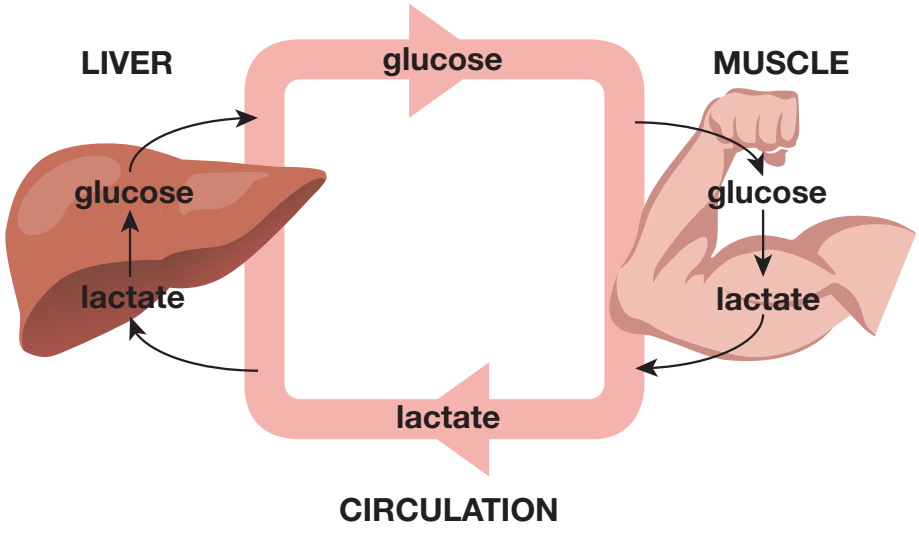


Figure 2.3: The Cori cycle. Pyruvate is generated from glucose as the end product of glycolysis. When pyruvate is reduced to lactate by the enzyme lactate dehydrogenase it can be released into the circulation, via which it reaches the liver. In the liver, lactate can be converted back into glucose. The formed glucose can then be excreted in the circulation again so that it's available for muscle tissue again. The Cori cycle is named after Carl Cori and Gerty Cori, the couple that discovered the cycle.

2.4 Oxidative energy system

Both the phosphagen energy system as well as glycolysis regenerate ATP without consuming any oxygen. However, the oxidative energy system, as the name suggests, does consume oxygen. This allows complete oxidation of the substrates, and thus allowing more energy to be extracted from each molecule of substrate. However, this doesn't mean that the power is high as well, this is lower than the other two energy systems (Table 2.1).

Additionally, the oxidative energy system can use fatty acids as a source of energy, unlike glycolysis. Fatty acids can only be burned aerobically. The oxidation of substrates takes place in the mitochondria instead of the cytosol where glycolysis takes place. Before fatty acids can be burned for energy, they must first be bound to carnitine in order to enter the mitochondria. This transport is facilitated by the mitochondrial carnitine palmitoyl transferase system, which is the flux-generating step in fatty acid oxidation [305].

The oxidative energy system can be divided into three steps. First, acetyl-CoA needs

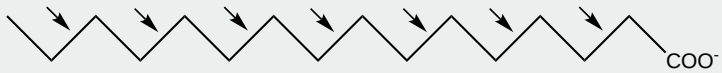
to be formed. Second, the formed acetyl-CoA is then fed into the citric acid cycle. And, finally, most of the energy is then released in the respiratory chain.

The first step thus involves the generation of acetyl-CoA from the substrate. When considering glucose metabolism, this boils down to converting pyruvate (provided by glycolysis) to acetyl-CoA by oxidative decarboxylation. This step is irreversible and is coupled to the reduction of an NAD^+ molecule (yielding NADH). This will later yield about 2.5 ATP in the respiratory chain. When considering fatty acids, β-oxidation is the first process taking place. In this process, an acetyl-CoA molecule is cleaved off from the fatty acid chain in four steps. Thus, the fatty acid chain is always shortened by two carbon atoms. Besides the truncated fatty acid chain and acetyl-CoA molecule this gives, NADH and $FADH_2$ are also produced. The produced NADH and $FADH_2$ together will yield 5 ATP for each round of β-oxidation of a fatty acid chain.

Box 2.2

The β-oxidation owes its name to the fact that it oxidizes the β-carbon atom of a fatty acid chain. The β-carbon atom is the second carbon atom when you start counting at the site of the functional group (the carboxyl group [COO^-] to which CoA is attached). The Greek alphabet is used to name the carbon atoms. For example, α- and ω-oxidation can also take place (although these don't play a significant role in energy metabolism).

In the image below, arrows highlight the C-C bonds of the fatty acid palmitate that are broken during β-oxidation. β-oxidation takes place 'from right to left' here. The two carbon atoms on the right are the first to be cleaved. A total of eight C2 units are formed by β-oxidation of this C16 fatty acid.



With α-oxidation the first carbon atom is oxidized, counted from the functional group, whereas with ω-oxidation the most distant carbon atom is oxidized (ω is the last letter of the Greek alphabet).

The well-known omega 3, omega 6 and omega 9 fatty acids also owe their name to this. For example, omega 3 fatty acids have their first double bond on the third carbon atom counted from the ω-carbon atom. Likewise, omega 6 fatty acids and omega 9 fatty acids have the first double bond on the sixth and ninth carbon atom, respectively, counted from the ω-carbon atom.

Glucose and fatty acid metabolism share the commonality that both generate the two carbon atom molecule acetyl-CoA. Acetyl-CoA forms the substrate for the citric acid cycle (described in Section 2.4.1), which ultimately yields potential energy to generate most of the ATP from a substrate. Although amino acids aren't commonly considered as a source of energy, they too are burnt to yield energy. It follows that, given that a human doesn't lose or gain net protein mass under normal circumstances, the amount of protein intake approximates the amount of protein (and thus amino acids) used to yield energy. Amino acids can be converted into glucose, fatty acids or ketones, after which they can supply energy by regular means. The amino acids that can be converted into glucose are called glucogenic amino acids and the amino acids that can be converted into fatty acids or ketones are called ketogenic amino acids. Some amino acids are both glucogenic and ketogenic.

Furthermore, amino acids can also directly provide energy because some amino acids can be converted into pyruvate and subsequently into acetyl-CoA. Examples include the

amino acids alanine, cysteine, glycine and serine. Still other amino acids can directly function as intermediate metabolites of the citric acid cycle such as succinyl-CoA. Examples are isoleucine, methionine and valine.

However, the citric acid cycle isn't the final destination yet. Although most of the potential energy is provided during the citric acid cycle, hardly any ATP is generated yet. The energy is disguised in molecules different than ATP, namely NADH and $FADH_2$. Most ATP isn't generated until the third and final step in which one can divide the oxidative energy system. The last step is known as oxidative phosphorylation and is mediated by the electron transport in the so-called respiratory chain. During the citric acid cycle, many energy-rich electron pairs are generated. These pairs are captured by the electron carrier molecules NADH and $FADH_2$. The respiratory chain releases the potential energy bit by bit to create a H^+ gradient along the inner mitochondrial membrane. The potential energy that is stored in this gradient is subsequently used by ATP synthase in the inner mitochondrial membrane, 'exploiting' the back flow of protons along their concentration gradient. ATP synthase kind of acts like a water wheel through which the protons flow. The energy this process yields is used to generate ATP again from ADP and P_i (hence the name ATP synthase). This entire process is called oxidative phosphorylation.

2.4.1 Citric acid cycle

The citric acid cycle (see Box 2.3) is an eight-step process in which an acetyl unit is completely oxidized to carbon dioxide and water. During these eight steps, energy is released and subsequently captured by the electron carriers NADH and $FADH_2$, as described earlier. Thus NAD^+ and FAD act as the electron acceptors for the citric acid cycle. And although there isn't direct generation of ATP during the citric acid cycle, an energy-rich phosphate is generated in the form of guanosine triphosphate (GTP). This molecule is then used pretty much instantly to rephosphorylate ADP to ATP, a reaction catalyzed by a nucleoside diphosphokinase. This reaction is as follows:

$$\mathrm{GTP} + \mathrm{ADP} \rightarrow \mathrm{GDP} + \mathrm{ATP}. \tag{2.4}$$

Box 2.3

i The citric acid cycle is also called the Krebs cycle, named after the biochemist Hans Krebs. Krebs, along with William Johnson, had discovered the cycle and won the Nobel Prize in Physiology or Medicine for it. Remarkably, Krebs' idea was first rejected by the highly prestigious scientific journal *Nature* due to lack of space. After this he submitted his work again to the scientific journal *Enzymologia*, where it was accepted quite quickly.

It isn't necessary to know all the steps of the citric acid cycle. The interested reader can look these up in pretty much every biochemistry textbook. This section will focus on the essence of the citric acid cycle.

It's important to realize that the citric acid cycle is central to the oxidative energy metabolism of all three macronutrients. Fatty acids, carbohydrates, and amino acids can all be used to provide energy through this pathway, because they can feed it the general substrate required for it (acetyl-CoA).

The net reaction of the citric acid cycle is as follows:

$$\begin{aligned} &3\ \mathrm{NAD^+} + \mathrm{FAD} + \mathrm{GDP} + \mathrm{P_i} + \text{acetyl-CoA} \\ &\rightarrow 3\ \mathrm{NADH} + \mathrm{FADH_2} + \mathrm{GTP} + \mathrm{CoA} + 2\ \mathrm{CO_2}. \end{aligned} \tag{2.5}$$

In addition to the acetyl unit, the energy carriers NAD, FAD and GDP are also required. The latter also requires P_i to be able to phosphorylate the energy-rich phosphate GTP. Most of the energy is ultimately carried by the nucleotide cofactors NAD and FAD. The energy stored in these molecules is later released in the respiratory chain and is used to form ATP. It should also be noted that oxygen isn't consumed yet in the citric acid cycle. This doesn't happen until later in the respiratory chain (see Section 2.4.2). Nonetheless, oxygen is required to keep the citric acid cycle going, otherwise the oxidized form of the required nucleotide cofactors will rapidly be depleted.

Box 2.4

Complete oxidation of the acetyl unit in the citric acid cycle is done in the absence of oxygen. The term oxidation can sometimes be confusing because it seems to imply the presence of oxygen. However, this doesn't always have to be the case. Besides the gain of oxygen by a molecule, any loss of electrons or hydrogen is also a form of oxidation. On the other hand, the loss of oxygen, or the gain of electrons or hydrogen by a molecule, is a form of reduction.

Oxidation cannot take place without simultaneous reduction of another molecule. When the acetyl unit is oxidized in the citric acid cycle, the nucleotide cofactors NAD^+ and FAD are reduced in concert. The reduced forms of these are NADH and $FADH_2$, respectively.

The potential energy stored in the produced NADH and $FADH_2$ is used in the respiratory chain to regenerate ATP from ADP. Each NADH molecule has the potential energy to regenerate about 2.5 ATP and each $FADH_2$ molecule to regenerate about 1.5 ATP. One full cycle yields 3 NADH, 1 $FADH_2$ and 1 GTP. This comes down to a total of 7.5 ATP for the 3 NADH molecules, 1.5 ATP for the $FADH_2$ molecule, and 1 ATP for the GTP molecule. Thus, summed up, 10 ATP for an acetyl unit.

Keep in mind, however, that glucose is split into two pyruvate molecules, so that the cycle is completed twice per glucose molecule. This brings the total to 20 ATP. In addition, the oxidative decarboxylation of pyruvate to acetyl-CoA also yields 2.5 ATP, as previously described. This also occurs twice per glucose molecule, thus yielding 5 ATP.

In addition to the 2 ATP generated during glycolysis, two molecules of NAD^+ are also reduced to NADH (see reaction 2.3). These two molecules do not hold the potential energy for 2.5 ATP, but only for 1.5 ATP per molecule. The reason for this is that the NADH is generated in the cytosol, where glycolysis takes place, and not in the mitochondria. The energy of the cytosolic NADH can be transferred to the mitochondrial respiratory chain, but this transport costs 1 ATP.

If we sum everything up, we arrive at 30 ATP for each molecule of glucose we burn. This is in stark contrast with the 2 ATP generated when it's solely anaerobically metabolized.

Box 2.5

Many textbooks report a total yield of 36 to 38 molecules of ATP instead of 30 for the complete metabolism of a glucose molecule. Although this

is theoretically correct, this number is somewhat lower in practice. The estimated amounts of ATP that are generated from the potential energy stored in NADH and $FADH_2$, as described in the text, more closely approximate the achieved yield in practice. The reason for this is that a large portion of energy is used for transport from the mitochondrial matrix to the cytosol. This transport is performed by the antiporter ATP/ADP translocase, that exchanges ATP for ADP from the cytosol.

2.4.2 Respiratory chain

The respiratory chain, also called the electron transport chain, consists of several proteins located in the inner mitochondrial membrane. These proteins cooperate to convert the potential energy of the energy carriers to ATP. The energy-rich electron pairs of NADH and $FADH_2$ travel along these proteins in the inner mitochondrial membrane by successive redox reactions. NADH and $FADH_2$ are oxidized during this process and as a result they are able to 'capture' electron pairs again from the citric acid cycle.

While the energy-rich electron pairs travel along the proteins of the respiratory chain, a little bit of energy derived from the electron pairs is used to pump proteins from the mitochondrial matrix to the intermembrane space (the space between the inner and outer mitochondrial membrane). At the end of this journey, when all energy has been extracted from the electron pairs, the electron pairs are used to reduce oxygen to water. By pumping protons from the mitochondrial matrix into the intermembrane space, an electrochemical gradient is established. Thus, the protons in the intermembrane space tend to flow back to the mitochondrial matrix. They flow back through specific channels in the inner mitochondrial membrane, which are designed to utilize the energy that comes free during this process in order to synthesize ATP. These channels are linked to the enzyme ATP synthase, which uses this energy to convert ADP and P_i into ATP. The ATP can then be exchanged for ADP by ATP/ADP translocase to get it into the cytosol.

Some of the generated ATP is also used to phosphorylate creatine to creatine phosphate in the mitochondria. Creatine phosphate then diffuses to the cytosol, where it can be used to rephosphorylate ADP to ATP, a reaction catalyzed by creatine kinase (see Reaction 2.2). This is called the creatine phosphate shuttle. This will mainly play a role in muscle fibers under conditions that are very energy-demanding, such as maximum contraction.

Part of the energy derived from the proton gradient is used to transport ADP to the mitochondrial matrix and ATP to the intermembrane space.

Finally, some of the energy is lost as heat due to 'leakage' in the inner membrane. Because of this leakage, some of the protons flow back without coupling of the energy this releases to ATP synthesis. This uncoupling is the result of so-called uncoupling proteins that are located in the inner mitochondrial membrane. The cutting agent 2,4-dinitrophenol (DNP) also uncouples the respiratory chain and has been used in the past to promote fat loss (see Box 2.6).

Box 2.6

In a 1933 study, Tainter et al. published their clinical results of the compound 2,4-dinitrophenol (DNP) for the treatment of obesity [436]. Their results were astounding, and it didn't take long before it became available without prescription in the United States. Losing your fat easily by popping a pill: it sounded too good to be true. And it was. In 1938, the drug was banned in the United States because it turned out to have very dangerous side

effects. Notable side effects are high fever (hyperthermia), increased heart rate (tachycardia), excessive sweating (hyperhidrosis), accelerated breathing (tachypnea), cataracts, and in some cases even death.

DNP works by uncoupling the respiratory chain, thus producing heat instead of ATP. It inhibits the uptake of P_i by the mitochondria (required for the synthesis of ATP from ADP and P_i by ATP synthase) and promotes 'leakage' of protons to the mitochondrial matrix as an ionophore.

The drug is still available on the black market and is sporadically used by bodybuilders and people who are looking for a quick fix to lose weight.

3. Absorption, metabolism and safety

3.1 Introduction

A supplement needs to reach its destination for it to be effective. A dietary supplement is taken orally, aka by mouth. Subsequently, the supplement is absorbed by the gastrointestinal tract and, usually, is carried to its destination by the blood circulation. Once it arrives at its destination, it can exert an effect.

During this journey to its destination, a supplement can be subject to metabolism. Metabolization is understood to mean biotransformation (chemical conversion) from the original molecule to another molecule (a metabolite). A metabolite of a supplement usually has different effects on the body than the original molecule. In some cases, the original molecule has no effect, and the supplement's action relies on biotransformation of the molecule into an active metabolite: bio-activation. In other cases, the action of a metabolite is similar to that of the original molecule. In most cases, however, biotransformation leads to deactivation. As a result, the molecule loses its function and its excretion from the body is promoted.

In this chapter, some of the concepts that lie at the root of absorption and metabolism of supplements will be highlighted. After all, absorption and metabolism are fundamental to the ultimate physiological effect of a dietary supplement. In addition to this intended effect, absorption and metabolism also play a crucial role in potential side effects that might result from its use. The topic of dietary supplement safety is briefly touched upon at the end of this chapter.

3.2 Absorption

As mentioned earlier, most supplements are taken by mouth. The supplement thus reaches the mouth first. When considering regular foodstuffs, such as rice, bread, or meat, the mouth doesn't play a direct role in the absorption of these products. The mouth then mainly functions as a grinding and 'inspection' machine. Grinding enlarges the surface of the ingested food considerably, which later has a beneficial effect on absorption. Additionally, both taste and smell play an important role: spoiled foodstuffs can be quickly avoided. However, supplements are commonly available in capsule, tablet, powder or ready-to-drink form. It's exceedingly rare that you actually need to chew on a supplement. Taste is enhanced by the addition of flavor additives in the case of most supplements that are distributed as powders or ready-to-drink liquids.

After a supplement has been in your mouth, it passes through the esophagus to the stomach. The stomach wall is made up of muscle layers that run in different directions. These muscle layers are responsible for mixing the stomach contents. Moreover, the stomach harbors a particularly acidic environment and contains the protein-splitting enzyme pepsin. This leads to rapid denaturation (the unfolding of the spatial structure) and degradation of proteins into smaller peptides.

The acidic environment can sometimes already lead to spontaneous degradation, i.e., non-enzyme-catalyzed degradation, of a supplement. For this reason, it's sometimes recommended to take certain supplements fasted. When a supplement is taken fasted, it will pass the stomach more quickly. Or in other words: it'll stay in the stomach for a shorter period of time. This short residence time can promote bioavailability.

The stomach usually doesn't play a noteworthy role with regard to the direct absorption of nutrients. Few things are significantly absorbed by the stomach wall. Some exceptions to this are alcohol and caffeine. For example, about 20 % of ingested caffeine is absorbed by the stomach [96].

Box 3.1

The supplement Kre-Alkalyn, a creatine variant, contains sodium carbonate. Sodium carbonate can function as a pH regulator and thus is added by the manufacturer to counteract degradation in the stomach due to its acidic environment—or at least that's the claim. Creatine supposedly would degrade to the useless metabolite creatinine at low pH levels. In the acidic environment of the stomach the sodium carbonate reacts with the hydrogen ions. This results in the formation of sodium ions, water (H_2O) and carbon dioxide (CO_2). This will lead to a slight increase in the pH value in the stomach, and thus making it less acidic.

However, it's a myth that creatine is significantly degraded at low pH values. The half-life of creatine to creatinine is 55 days at a pH value of 1.4, 7.5 days at a pH value of 3.7, and 40.5 days at a pH value of 6.8 [350]. Since the residence time in the stomach is a fraction of these half-lifes—with the longest half-life even being measured at a pH value of 1.4—it's pointless to 'protect' creatine against low pH values.

Leaving the stomach, a supplement enters the small intestine. The first part of the small intestine is called the duodenum. The pancreas serves to neutralize the low pH value of the mixture (chyme) that has arrived from the stomach by adding hydrogen carbonate (bicarbonate). Additionally, the pancreas secretes various zymogens. These zymogens are the precursors of active enzymes (peptidases) which can further break down peptide chains into free amino acids, di- and tripeptides. Bile is also added here. The bile is responsible

for emulsifying lipids, thus creating many very small lipid droplets. This causes a large increase in the surface area of these lipids, which lipases—enzymes that break down fats into their fatty acid constituents—can act on and thus promote their absorption. Addition of bile salts further enhances the absorption of small lipophilic molecules by the intestinal wall.

The small intestine has a huge surface area to aid in absorption. This large surface is established by its length (the small intestine is several meters long) and, more importantly, the intestinal wall is strongly folded. The cells of the intestinal wall are arranged in characteristic finger-like projections (villi). Interestingly, the cell membrane of enterocytes on the apical side (the side facing the inner side of the intestine) also contains finger-like protrusions (microvilli) which further increases the surface area.

The enterocytes, the cells that line the inner wall of the small intestine, are responsible for the absorption of molecules. Several transport mechanisms play a role in this. These transport mechanisms can be roughly divided into two groups: paracellular transport and transcellular transport.

Paracellular transport means that the molecule is taken up by slipping through the space between two neighbouring enterocytes. As it happens, there is a very small space between two enterocytes called the zonula occludens, or perhaps better known as a tight junction. In principle, only small molecules find their way between the enterocytes. Additionally, the surface area of this space is a fraction of the surface area formed by the membranes of the enterocytes. As such, this mechanism of transport generally has no significance in the absorption of a supplement that is also taken up transcellularly.

In transcellular transport the molecule crosses the membrane of the enterocyte. First, transport takes place across the apical membrane (The membrane facing the inside of the intestine). It then moves through the cell to the opposite side, where it crosses the membrane again and enters the interstitial fluid and blood vessels leading to the liver. Transport across the membrane can be performed both actively and passively. Active transport requires energy that the cell must provide, while passive transport can take place without the cell having to provide energy for it.

Finally, food residues can end up in the large intestine. The large intestine is especially important for the absorption of water and the digestion of residual carbohydrates (dietary fiber and resistant carbohydrates in particular) that have escaped the small intestine. The large intestine contains many bacteria that can further digest these ‘leftovers’. The bacterial digestion of carbohydrates yields short-chain fatty acids. These are then absorbed by the intestinal epithelium and can then be used to provide energy. The role of the large intestine is quite limited in regard to dietary supplements. An interesting exception is hydroxypropyl distarch phosphate (HDP) (see Box 3.2).

Box 3.2

Hydroxypropyl distarch phosphate (DHP) is added to foods as a thickener or binding agent and is a resistant type of starch. It hardly gets digested in the small intestine. It gets partially fermented into short-chain fatty acids by bacteria in the large intestine. Some of these fatty acids are then used by these resident bacteria and the remainder gets absorbed through the intestinal wall. Clinical research demonstrates that HDP ingestion leads to a strong increase in energy expenditure compared to ingestion of a regular carbohydrate (waxy maize) [416]. It’s said that it also satiates well. For these reasons, some dietary supplements are available that contain HDP. These are commonly

marketed to aid in fat loss.

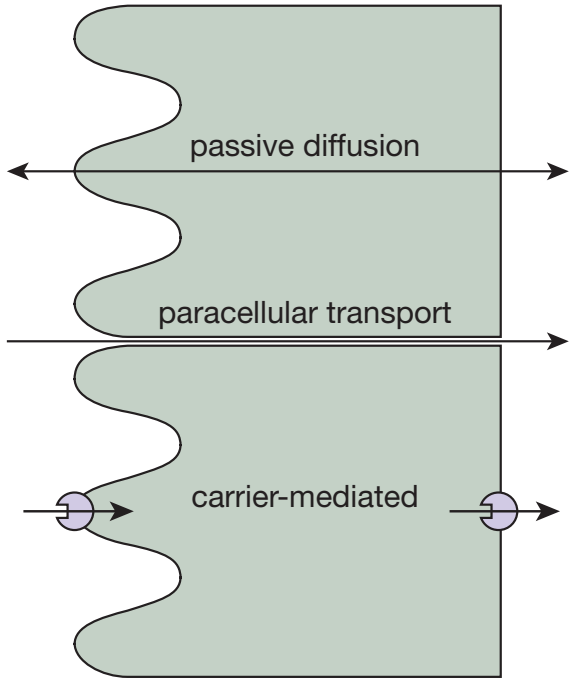


Figure 3.1: Different modes of transport. See text for further information.

3.2.1 Passive transport

In passive transport, transport takes place across the membrane without the cell needing to (directly) supply energy for it. The transport is driven by a concentration gradient of the molecule in question. This means that in passive transport, the net movement of a substance is always from an area of high concentration to an area of low concentration of the substance. Therefore, passive transport never occurs in the opposite direction of low to high concentration.

Passive transport can take place in two ways. The simplest way is by simple diffusion, in which a substance freely diffuses through the phospholipid bilayer of a (cell) membrane. However, not all substances have the physicochemical properties to diffuse freely across the phospholipid bilayer, such as glucose. For some of these substances, the cell membrane is equipped with special transporters to still allow diffusion of them. When such a transporter is used to get a substance to cross the cell membrane, it's called facilitated diffusion.

Passive transport by diffusion can be described by Fick's first law of diffusion. Fick's first law of diffusion is described as follows:

$$I_d = D_m \cdot \frac{\Delta C \cdot O}{d_x} \text{mol/s}. \tag{3.1}$$

On the left side of the equation is the diffusion flux (I_d; the number of molecules that diffuse across the membrane [expressed in mol/s]). On the right side of the equation you can see D_m, the membrane diffusion coefficient (in cm^2/s); ΔC, the difference in concentration between both sides (in mol/cm^3); O, the membrane surface area (in cm^2); and d_x, the membrane thickness (in cm).

The membrane surface area O and membrane thickness d_x speak for themselves. The larger the membrane surface across which the molecules can diffuse, the higher the diffusion flux I_d will be. And the thicker the membrane across which a substance has to diffuse, the slower the diffusion takes place.

The membrane diffusion coefficient D_m deserves some explaining. It's dependent on various things and is highly dependent on the type of molecule involved. D_m is determined by the temperature, molecular mass, viscosity, and permeability of the membrane to the molecule. It's more difficult for a large molecule to diffuse across the membrane than a small molecule. Likewise, a charged molecule diffuses more difficultly across the membrane than an uncharged molecule. Since the cell membrane is comprised of a phospholipid bilayer, the molecule must also have some lipophilicity to cross it. The membrane is virtually impermeable (pervious) to purely hydrophilic substances, unless specific transporters are present in the membrane for the substance in question. When these transporters merely facilitate diffuse and don't consume chemical energy, either directly or indirectly, it is facilitated diffusion. However, facilitated diffusion doesn't follow Fick's first law of diffusion, but can be described according to Reaction 3.2 as described in Section 3.2.2.

3.2.2 Active transport

Active transport differs from passive transport in that the cell must provide energy for transport to take place. In passive transport, the energy comes from the concentration gradient and the net molecular flux follows the gradient from a high concentration to a low concentration. However, with active transport, molecules can be transported against the concentration gradient. This requires energy, since the energy will not be derived from the concentration gradient. Thus, in active transport, transport commonly takes place from a low concentration to a high concentration—in contrast to passive transport.

Although it's not necessary for this type of transport to move from a low concentration to a high concentration, this nevertheless is often the case in practice. A notable example is the sodium-dependent glucose cotransporters that are present in the enterocytes of the small intestine and the nephrons of the kidneys. It's important to absorb as much glucose from the intestines as possible to prevent energy from being lost because it isn't being absorbed fully. The energy required for active transport of these glucose molecules is insignificant compared to the energy that they can eventually provide. It's also important not to lose glucose, and therefore energy, in the urine. In addition, the body will lose significant amounts of fluids if the nephrons would fail to reabsorb the glucose due to the osmotic gradient in the filtrate it'll cause.

Active transport couples transport to a reaction that provides the energy to achieve this. ATP is, with few exceptions, always the source of energy for this. A well-known example for this is the sodium potassium pump that maintains the low intracellular sodium concentration and high intracellular potassium concentration. The sodium potassium pump transports sodium out of the cell and potassium into the cell, both against their concentration gradients. The transport is coupled to the energy-yielding reaction of ATP

hydrolysis.

A transport protein can also obtain its energy indirectly from ATP hydrolysis. In many cases, the transport of two molecules goes hand in hand. In such cases, it's possible that one of the molecules supplies the energy required for the active transport of the other molecule, because it flows down its concentration gradient. For example, there is a very low sodium concentration intracellularly compared to extracellularly (outside the cell). The flow of sodium down its concentration gradient, i.e., from extracellular to the cytoplasm, which yields energy, can then be coupled to the active transport of another molecule against its concentration gradient. Thus in such a case the energy is sourced from the sodium concentration gradient. This concentration gradient, in turn, is maintained by the action of transporters that actively pump sodium out of the cell, such as the aforementioned sodium potassium pump, which gets its energy from ATP hydrolysis to do so. Thus, indirectly the energy derived from ATP hydrolysis is coupled to the active transport of a molecule. This is also called secondary active transport.

It's called carrier-mediated transport when a molecule is transported across the membrane by a transporter. The rate at which this takes place is limited by the transport capacity of the specific transport proteins which do so. Such transport can be described by the following equation:

$$I_a = \frac{T_{max} \cdot C}{K_d + C}. \tag{3.2}$$

On the left side of the equation is the active transport flow (I_a) (the number of molecules that are moved across the membrane per second). On the right side of the equation are the maximum transport capacity of the transport protein system T_{max}, the concentration C of the molecule that is to be transported, and the dissociation constant of the transport protein molecule complex K_d (expressed as a concentration).

When C equals K_d the half-maximum rate of transport is reached, i.e. $0.5 \cdot T_{max}$.

This brings us to some important differences between a passive diffusion flow across a membrane and a carrier-mediated flow. When considering a passive diffusion flow, the flow is proportional to the concentration difference ΔC between both sides of the membrane. This is in contrast with a carrier-mediated flow, where it will only move proportional to C within a limited interval of concentrations. Transport will approximate T_{max} once the concentration greatly exceeds K_d. The transport is therefore limited, in other words: the transport system can become saturated, such that the rate of transport does not increase further beyond a certain concentration. At a certain concentration (almost) all transport proteins will simply be busy transporting another molecule across the membrane, such that a higher concentration has no further effect on it.

The T_{max} of a transport protein system can be regulated by the cell, as is the case with the GLUT family that facilitates glucose transport across the membrane. Insulin triggers the translocation of GLUT4 to the cell membrane, thereby increasing T_{max}; a form of up-regulation. Analogously, transporters can also be removed from the membrane (down-regulation). There is also the possibility of post-translational modifications to the transport proteins, such as phosphorylation, which influences K_d. The take-home message here is that carrier-mediated transport can be actively regulated by the cell in several ways, and that it can become saturated at a certain concentration so that transport doesn't proceed faster with a further increase in concentration (in contrast to passive diffusion, where the transport is proportional to the concentration).

Although carrier-mediated transport is discussed here in the section about active transport, carrier-mediated transport can also facilitate passive transport. This is called facilitated diffusion (as mentioned earlier in Section 3.2.1). However, it's discussed here because active transport is always carrier-mediated and carrier-mediated transport has the same kinetic characteristics regardless of whether it's passive or active.

3.3 Metabolism

Metabolism of a substance can already start to take place right after ingestion. That is, biotransformation can take place. Biotransformation means another molecule (or molecules) is formed. This is catalyzed by enzymes that can be found all throughout the body.

With dietary supplements, the 'purpose' of metabolism of a molecule can be a number of things. For example, the purpose might be to convert the molecule into a suitable form for usage by the body, such as with degradation of polysaccharides into monosaccharides, or to produce a biologically active metabolite by chemical modification (bio-activation). Additionally, the purpose might be to 'disable' a molecule and promote its excretion so that it doesn't accumulate in the organism with the potential of causing harm. (Although this mainly plays a role with drugs and xenobiotics. Many dietary supplements are already hydrophilic and can often be easily excreted without further biotransformation). The latter is usually divided into phase I and phase II metabolism. Together, these phases—generally—ensure that a molecule becomes more water-soluble (more polar). This allows it to be excreted more easily in the urine by the kidneys.

Phase I metabolism prepares the molecule for phase II metabolism (although phase II metabolism certainly doesn't always have to succeed phase I metabolism). It does so by forming a functional group such as a hydroxyl group (-OH), thiol group (-SH), amine group (-NH_2) or formyl group (-CHO). Some types of reactions that the body uses for this purpose are oxidation, reduction, hydrolysis, hydroxylation, desulphation, deanimation, dehydrogenation and dealkylation.

Following phase I metabolism, polar groups can be conjugated to the molecule (phase II metabolism). Central to this are sulfation and glucuronidation, in which a sulfate group and a glucuronic acid, respectively, are conjugated to the molecule. Acetylation, methylation and binding the molecule to an amino acid also take place.

To illustrate this process, we can have a look at the metabolism of vitamin D, which is commonly supplemented in the form of cholecalciferol (vitamin D_3). Vitamin D_3 is quite apolar/lipophilic and is absorbed by passive diffusion (and also by cholesterol transporters [375]) by the enterocytes in the small intestine. In the enterocytes, a large part of the vitamin D_3 is then packed into chylomicrons with other lipids, after which it enters the circulation via the lymphatic system and then reaches the various tissues. However, vitamin D_3 itself has no biological activity. The molecule must first be converted (biotransformation) into an active molecule (bio-activation). To this end, first, hydroxylation (addition of a hydroxyl group [-OH]) takes place at carbon atom 25. This reaction occurs in the liver and yields 25-hydroxyvitamin D (25[OH]D). A second hydroxylation step takes place in the kidneys, this time at the first carbon atom. This yields 1,25-dihydroxyvitamin D (1,25$[OH]_2$D). 1,25$(OH)_2$D is the biologically active molecule.

The degradation of 25(OH)D and 1,25$(OH)_2$D primarily takes place by hydroxylation at carbon atom 24. The production of this reaction, calcitroic acid in the case of hydroxylation

of $1,25(OH)_2D$, is already quite water-soluble. This allows it to be more easily excreted in the urine by the kidneys. Glucuronidation of $1,25(OH)_2D$ can also take place [198]. This metabolite can be excreted in the bile, after which reabsorption in the small intestine can take place (enterohepatic cycle).

3.4 Safety

Dietary supplements aren't subject to the same rigorously conducted clinical trials as are drugs. As a result, relatively little clinical data is available, especially for the somewhat newer supplements. This often makes it difficult to draw well-founded conclusions about the safety of such a supplement. In addition to the fact that most clinical trials with dietary supplements have a low number of subjects, these trials usually are quite limited as well in examining potential harmful effects. After all, the budget for such studies is usually quite limited. Consequently, because there's usually little research into the safety of dietary supplements, this also simply tends to receive less attention. Notwithstanding that safety is, of course, an important aspect in the decision to supplement something or not.

Safety of food and dietary supplements is an incredibly broad subject with lots to write about. This section, however, will only briefly touch on the matter, providing a few useful take-home messages.

Safety studies can be classified in a number of ways. One way is to classify these based on the test model that was used: cells, animals or humans. Cell studies give an idea of the effect of a dietary supplement on a very small scale, namely that of the cell. These types of studies are particularly useful for uncovering possible mutagens (substances that can damage DNA) or, for example, the effect on the activity of certain enzymes. Important with these types of studies is the cell type that is used, but of course also the concentration of the substance under investigation. Cell studies obviously have many limitations and should never be leading in judging the safety of a supplement. It does, however, provide useful information that can be used in follow-up research in animals or humans, in order to pay more attention to, for example, a particular organ or harmful effect.

Animal studies allow to investigate harmful effects in an organism. During an experiment, samples can easily be collected from animals and organs can be dissected and examined after death. Animal studies, if done correctly, hold some predictive value for what might happen in humans. However, research in animals is plagued by various issues—it is thus by no means always carried out correctly or extrapolatable to humans. An important point is that a correct animal model must be picked, which is often not easy when little is known about the substance in question. It's very common to see rodents such as rats or mice to be used for animal studies, simply because they have many practical advantages. For example, they are relatively easy to research, a lot is already known about these rodents, and there are a great many different strains and genetically modified variants available to choose from. They are also relatively inexpensive and arguably most researchers have fewer ethical problems with conducting experiments on a rat or a mouse than, say, a primate. An important consideration when electing the right animal model is the similarities between the animal and humans in terms of absorption, distribution, metabolism and excretion (ADME) of the substance. Very often, differences between animals and humans are—from my experience—seemingly caused by differences in the metabolism of a substance. Nevertheless, without in-depth knowledge of animal experiments, it's often difficult to assess whether an animal model was appropriately selected

and executed.

Clinical studies in which the potentially harmful effects of a substance are investigated in humans remain leading. Because researchers are dealing with, among other things, financial limitations, they have to make concessions on what they do and do not research during a clinical trial. Results from previous cell experiments and animal studies can aid in paying attention to certain things. If animal studies show that a substance affects a particular organ, it's logical to keep an eye on this organ during clinical research. For example, if the liver has been affected in animal experiments, you'll pay extra attention to the liver. This can then be done in several ways. A cheap yet fairly reliable method to test the status of the liver is by a simple blood test. You can measure certain blood values that say something about the liver. A brief overview of some blood values and what they relate to is given in Table 3.1.

Blood values	**Says something about**	**Remarks**
ALAT, ASAT, GGT, AF	Liver damage	ALAT and ASAT can be increased by resistance exercise (see Box 9.2) .
Creatinine, GFR, Urea	Kidney function	Creatinine can be increased by creatine supplementation or large muscle mass (see Box 8.3). Urea can also be increased by a protein-rich diet.
Leukocytes	Immune system	
Hemoglobin	Oxygen transport	
Cholesterol, triglycerides	Cardiovascular disease risk	Values are strongly affected by diet.

Table 3.1: Overview of some commonly measured blood values. Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GGT, gamma-glutamyltransferase; AF, alkaline phosphatase; GFR, glomerular filtration rate.

In addition to objectively identifying harmful effects by a researcher, for example through blood tests, it's also important that test subjects are encouraged to report side effects themselves. This is because a side effect doesn't necessarily need to express itself to the researcher by the tests that are being performed. However, any side effect that occurs during the supplementation period doesn't necessarily have to be caused by the supplement itself. To determine this, a statistically significant difference compared to the placebo group needs to be found. A problem with this is that many clinical trials have a low number of test subjects, so that a type II error can easily occur. An additional disadvantage of small-scale studies is that the chance is very small that a rare side effect will be detected. After all, if a study doesn't find any side effects, that doesn't mean the supplement doesn't have any. The maximum risk (with a confidence interval of 95 %) of side effects can be approximated using the equation $\frac{3}{n}$, where n is the number of subjects [192]. For example, if no side effects have been observed in fourteen test subjects, the maximum risk is still approximately 1 in 5.

4. Molecular mechanisms of muscle growth

4.1 Introduction

The field of molecular exercise physiology is a relatively young subspecialty within exercise physiology. A lot of new developments have taken place in the field in recent years. In the 1970s and 1980s many new research methods in molecular biology popped up. For example, being able to manipulate the DNA of living organisms so that transgenic animal models could be developed. Genes could be selectively added (knock-in) or turned off (knock-out). By observing the effects of this, it can be reasoned in which processes a particular gene is involved.

A famous example in the context of muscle growth is that of the myostatin knock-out mouse by McPherron et al. [307]. The researchers disabled the myostatin gene in mice. As a result, the mice had a greatly increased muscle mass compared to normal mice, which was due to both hypertrophy and hyperplasia. As such, this research told us that myostatin is an important negative regulator of muscle growth.

Besides methods that enable manipulating the genome, other methods have also been developed that enabled to measure what goes on in a cell. Consider, for example, the development of the so-called western blot technique (see also Box 1.4) that allows for the detection of proteins. Or the detection of RNA by means of reverse transcriptase polymerase chain reaction (RT-PCR), which superseded the so-called northern blot—allowing to map changes in gene expression.

And like this, many other analytical techniques can be thought of that pushed the evolution of molecular exercise physiology forward. Through the efforts of many researchers that have applied these techniques in the context of muscle growth, much knowledge has been gained about the molecular interplay that leads to an increase in muscle size.

The effects of diet and exercise are, ultimately, mediated by signal transduction. The muscle cells receive signals that are interpreted by the cell and eventually lead to a response, either muscle hypertrophy or muscle atrophy. These can be signals about the availability of energy in the form of glycogen or ATP. But they can also indicate whether there are enough nutrients available for actual protein synthesis, i.e., amino acid availability. The mechanical stimulus triggered by muscle contraction, or a circulating hormone such as testosterone, also acts as a signal that can be picked up by the muscle cell.

Protein sensors of the muscle cell pick up these signals and then interpret them. This interpretation is carried out by a complex interplay of proteins that transduce the signals through the cells and, in a way, bring them together. Often times, the same proteins play a role in mediating this signal and converging it to the same point. Together, these proteins and the passing of signals among them, form so-called pathways.

The following section describes such a pathway. One that is closely involved in muscle growth. The proteins that are a part of such a pathway 'calculate' the final reaction that's about to take place by means of various mechanisms. This includes protein-protein interactions, post-translational modifications (e.g. phosphorylation), translocation (e.g. movement from the cytosol to the nucleus), and affecting the synthesis/breakdown of signaling molecules involved in the pathway. Ultimately, this causes changes in cellular processes such as gene expression and mRNA translation, but also protein breakdown. For example, changes in the concentration of metabolic enzymes or of the proteins that shape the sarcomeres and thus influence the functioning of the muscle. Research focused on muscle hypertrophy mainly looks at the effects on protein synthesis and gene expression of myofibrillar proteins and proteins involved in muscle breakdown, such as ubiquitin ligases.

4.2 mTOR signaling

The mechanistic target of rapamycin (mTOR) pathway plays a pivotal role in the intracellular signaling of muscle growth [59]. mTOR signaling gets its name from mTOR, a protein kinase involved in the phosphorylation of molecules that regulate protein translation initiation. To fulfill its role, the kinase forms a protein complex (mTORC1) with other proteins: mLST8 and raptor [127].

mTORC1 is known as a very important signaling complex that controls muscle mass [5]. It integrates the signals of training (mechanical and metabolic stress), amino acid availability, energy availability (cellular energy status) and growth factors. These signals can activate mTORC1. This results in phosphorylation of two major sets of substrates: eIF4E binding proteins and ribosomal S6 kinases. Phosphorylation of these substrates subsequently promotes protein synthesis, in particular its initiation.

Box 4.1

Protein synthesis (mRNA translation) can be subdivided into three phases: initiation, elongation and termination. Initiation forms the beginning of mRNA translation. A protein complex (small ribosomal subunit) binds to the 5'-cap of the mRNA molecule and then searches for the start codon to initiate translation of the mRNA to protein, starting with the first amino acid (methionine). The protein complex is assisted with its search for the start codon by several factors (eukaryotic initiation factors). Once arrived at the start codon, the initiation factors dissociate and the large ribosomal subunit joins the small ribosomal subunit to form a fully functioning ribosome. The

process by which the amino acids are then chained one by one to the growing polypeptide strand by the ribosome is called elongation. Once arrived at the stop codon, termination begins and the entire complex dissociates from the mRNA.
The initiation phase in particular is highly subject to regulation by anabolic pathways in muscle cells. For example, mTORC1 phosphorylates the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [164]. 4E-BP1 binds to one of the eukaryotic initiation factors (eIF4E) that causes the small ribosomal subunit to bind the 5' cap of the mRNA molecule. Binding of 4E-BP1 to eIF4E inhibits this recruitment of the small ribosomal subunit and thus inhibits mRNA translation. Phosphorylation of 4E-BP1 by mTORC1 leads to dissociation of the protein, thus allowing mRNA translation to proceed.

4.2.1 Regulation by growth factors

The regulation of mTORC1 by the growth factors insulin and insulin-like growth factor 1 (IGF-1) has been mapped out well. Activation of the insulin receptor or insulin-like growth factor 1 receptor (IGF-1R) allows for interaction and phosphorylation of the insulin receptor substrates (IRSs). Both the IR and IGF-1R are so-called tyrosine kinase receptors: they possess intrinsic kinase activity to phosphorylate proteins on tyrosine residues. Tyrosine phosphorylation of the IRSs creates binding sites on these proteins. Other proteins that contain the corresponding protein domain can then dock to it. In this case, it's the SH2 domain.

One of these proteins that has such a domain is phosphatidylinositol-3 kinase (PI3K). PI3K is subsequently activated after docking to an IRS [323]. Activated PI3K phosphorylates inositol phospholipids (located in the cell membrane) at the hydroxyl group (-OH) on carbon atom 3 to yield 3'-phosphoinositides, including phosphatidylinositol (3,4,5)-triphosphate (PIP3). Proteins that contain the Pleckstrin homology (PH) domain can interact with PIP3 and are thus recruited to the cytosolic side of the plasma membrane. This includes 3-phosphoinositide dependent protein kinase 1 (PDK1) and Akt (also known as protein kinase B). The association of PIP3 with Akt allows it to be more easily activated by phosphorylation. Additionally, the association of PIP3 with PDK1 causes it to phosphorylate Akt.

The Akt family of proteins consists of three isoforms, or three different kinases that catalyze the same reaction, but differ in their amino acid sequence. The three isoforms are Akt1, Akt2, and Akt3. In the context of muscle growth, only the first two are of interest, since Akt3 is not expressed in muscle tissue [487]. PDK1 phosphorylates Akt1 and Akt2 at residue Thr308 and Thr309, respectively. However, full Akt activity also requires phosphorylation on a serine residue [9, 150], namely Ser473 and Ser474 on Akt1 and Akt2, respectively. This phosphorylation is (probably) done by mTORC2 (a complex that also contains the mTOR kinase) [401].

Mechanistic studies often look at the phosphorylation status of one of both these residues to get an idea of Akt activation. These studies usually look at the phosphorylation status of Akt, thus looking at phosphorylation of residues Thr308 and Ser473.

Myostatin, a potent negative regulator of muscle growth [387], also regulates Akt activity [445]. Myostatin belongs to the transforming growth factor β (TGF-β) super family and is a ligand for activin type II receptors (ActRIIA and ActRIIB). The protein and its function were discovered in 1997 by McPherron et al. [307]. They turned off the gene for myostatin in mice and saw that they became very muscular. Their enormous muscle growth wasn't only due to an increase in the size of muscle cells (muscle hypertrophy), but

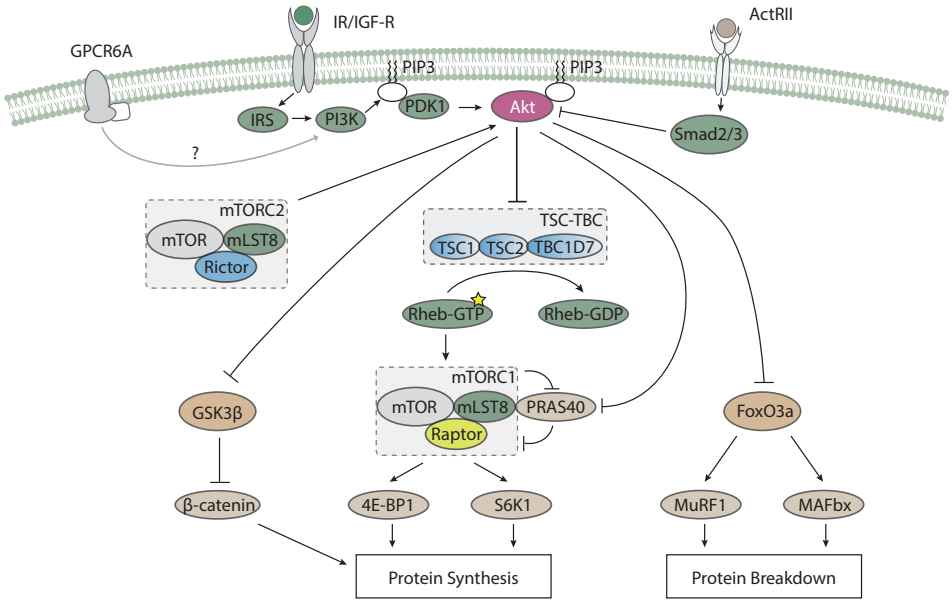


Figure 4.1: mTORC1 regulation by growth factors. Activation of the IR and IGF-1R causes the IRSs to be phosphorylated and thus activated. This leads to activation of PI3K. PI3K then generates PIP3, which recruits PDK1 and Akt to the plasma membrane. At the plasma membrane, Akt can be activated. This happens by phosphorylation by both PDK1 and mTORC2. Activated Akt inhibits several substrates by phosphorylating them, including the TSC-TBC complex, GSK3β, FoxO3A, and PRAS40. Akt activity is also stimulated by androgens, possibly by enhancing PI3K activity, an effect mediated by GPCR6A. Finally, Akt activation is inhibited by activation of ActRII receptors, an effect mediated by Smad2 and Smad3. Figure taken from Bond [59]. Abbreviations: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; ActRII, activin type 2 receptor; FoxO3a, forkhead box O3a; GSK3β, glycogen synthase kinase 3β; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1; IGF-R, insulin-like growth factor; IR, insulin receptor; IRS, insulin receptor substrate; mLST8, mammalian lethal with SEC13 protein 8; mTORC1/2, mechanistic target of rapamycin complex 1/2; S6K1, S6 kianse 1; TSC-TBC, tuberous sclerosis-Tre2-Bub2-Cdc16.

also due to an increase in the number of muscle cells (muscle hyperplasia). Since then, an enormous amount of research into myostatin signaling has been conducted to explore if it's possible to develop a drug that exploits this pathway and thus can be used in treatment of syndromes in which muscle mass is at stake, such as in cancer cachexia and muscular dystrophy.

Recently it has been discovered how myostatin inhibits Akt activity. Under normal circumstances, a micro-RNA (miR-486) is present in muscle cells that promotes Akt phosphorylation by inhibiting phosphatase and tensine homolog (PTEN). PTEN abrogates the action of PI3K by dephosphorylation of PIP3 into PIP2 [293]. Myostatin inhibits the expression of miR-486 at the transcriptional level, thus effectively inhibiting Akt phosphorylation by PI3K [212].

Akt has different substrates that are phosphorylated by the kinase upon its activation. The most well-researched substrates are: glycogen synthase kinase 3β (GSK3β) [108], proline-rich Akt substrate of 40 KDa (PRAS40) [265], tuberous sclerosis complex (TSC2) [234], and the forkhead box class O (FoxO) family of proteins [444].

TSC2 and PRAS40 inhibit mTORC1 activity and when Akt phosphorylates these, it abolishes this inhibition. TSC2 inhibits mTORC1 activity by forming a protein complex together with TSC1 and the in 2012 discovered TBC1D7 [126]. TSC is the acronym for tuberous sclerosis, a disease in which TSC1 or TSC2 is mutated, which means that its inhibitory effect on mTORC1 is gone. This disease is characterized by the proliferation of benign tumors in various organs, which is indeed in line with what is known about the central role mTORC1 plays in a cell: stimulating growth and proliferation. When the TSC complex is formed, it inhibits mTORC1 activity through its GTPase activating protein (GAP) domain [279, 126]. This is because mTORC1 is activated by GTP-bound Rheb proteins (Rheb-GTP) at the lysosomal surface [284]. By regulating the amount of Rheb-GTP, the TSC complex regulates mTORC1 activity. However, it should be noted that the TSC complex demonstrates its highest affinity for Rheb-GDP instead [309] and thus may hinder the exchange of guanosine diphosphate (GDP) for guanosine diphosphate (GTP) in particular. Which basically means that the Rheb proteins cannot 'recharge' with GTP.

As mentioned, phosphorylation of TSC2 (on Ser939 and Thr1462) by Akt inhibits its inhibitory effect on mTORC1. Several hypotheses have been postulated to explain why this phosphorylation inhibits the TSC complex. One of them suggests that the phosphorylation would lead to a more difficult formation of the TSC complex or reduce its stability. However, recent research shows that this is not the case—at least until twelve hours after stimulation of the PI3K-Akt pathway [309]. Additionally, this same study also showed that there was no decreased GAP activity of the TSC complex, which had also been previously suggested as a potential mechanism. Instead, the researchers describe a very tempting alternative hypothesis. Phosphorylation of the TSC complex would cause it to dissociate from the lysosomal membrane, the site where mTORC1 is activated by Rheb-GTP. This spatial regulation is currently the leading hypothesis with regard to how the TSC complex is regulated by Akt.

Box 4.2

Until recently, lysosomes were viewed as oval organelles that exist in the cytoplasm of cells. However, it has recently been discovered that lysosomes form a tubular network in the muscles of *Drosophilia* (fruit flies, a model organism which is widely used in biological research) [241]. Defects in this tubular organization seem to underlie several muscle diseases. However, the further implications of this discovery are still in their infancy.

mTORC1 activity is also inhibited by the binding of PRAS40 to the complex [473]. This protein binds to the mTORC1 subunit raptor, thereby disabling its ability to bind its substrates. Growth factor receptor activation causes PRAS40 to dissociate from mTORC1, thus alleviating this inhibition. The dissociation is caused by phosphorylation of the protein on three residues; one threonine residue (Thr246) and two serine residues (Ser181 and Ser221). The threonine residue is phosphorylated by Akt and the serine residues are both phosphorylated by mTORC1 when activated, such as by Rheb-GTP.

Phosphorylation of GSK3β, also a substrate of Akt, results in accumulation of β-

catenin in the cytoplasm. GSK3β normally ensures that β-catenin is broken down by forming a complex with other proteins [233]. When GSK3β is phosphorylated by Akt, this doesn't happen. β-catenin then translocates to the nucleus, where it affects gene transcription of genes involved in growth [19]. Additionally, GSK3β can inhibit mRNA translation by blocking the GDP-GTP exchange of eIF2B [366], which is required for the formation of a functional protein translation complex [160].

The FoxO family of proteins, on which Akt exerts an inhibitory effect, play a very important role in the breakdown of protein. They modulate the ubiquitin-proteasome proteolytic pathway, as well as autophagy of proteins [399]. The ubiquitin-proteasome system in particular plays an important role in muscle protein breakdown. This system functions by tagging proteins for degradation with a tag (ubiquitin). So-called ubiquitin ligases place these markers on the proteins. A large protein complex (the proteasome) then recognizes these markers, after which it breaks down the tagged protein. Two important ubiquitin ligases that have been subject of much research are muscle atrophy F-box (MAFbx/Atrogin-1) and muscle ring finger 1 (MuRF1) [56, 55].

Finally, Akt activity is also under the regulation of androgens [59]. This effect appears to be mediated by the G-protein coupled receptor GPCR6A, as well as by the intracellular androgen receptor (AR). These receptors likely interact with PI3K.

4.2.2 Regulation by energy status

The integration of cellular energy status is done by AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric protein consisting of a combination of α-, β- and γ-subunits. Of the α- and β-subunit, two isoforms of each are known. (α1, α2 and β1, β2). Three isoforms are known of the γ-subunit (γ1, γ2, γ3). The α-subunit functions as the catalytic unit, whereas the other two subunits play a regulating role and 'sense' the energy status of the cell. For example, the β-subunit can interact with glycogen [303] and the γ-subunit with the nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) [193].

A decrease in the energy status of the cell (that is, a decrease in glycogen or an increase in the ADP:ATP or AMP:ATP ratio) activates AMPK. In general, activation of AMPK promotes catabolic pathways to generate energy and thus restore energy status. In addition, it leads to inhibition of anabolic pathways in order to reduce energy expenditure. By means of the β-subunit, AMPK can integrate the status of the glycogen energy reserve. The interaction between both leads to allosteric inhibition of AMPK activity. A decrease in glycogen decreases this inhibition and thus leads to activation of the complex. This makes sense, as a drop in glycogen indicates energy expenditure. In addition, AMPK interacts with the nucleotides in order to 'measure' the current energy status of the cell. Competition between the three nucleotides ATP, ADP and AMP takes place to bind to the γ-subunit. A decrease in ATP and an increase in AMP and AMP are indicative of energy expenditure (see Chapter 2) and thus competition between these nucleotides for binding to the γ-subunit enables the complex to measure energy expenditure.

Of the twelve different combinations that can be made from the different subunit isoforms, only three of these have been found in human muscle cells (quadriceps): $\alpha2/\beta2/\gamma1$, $\alpha2/\beta2/\gamma3$ and $\alpha1/\beta2/\gamma1$ [46]. The distribution of these three heterotrimers is estimated to be 15 % $\alpha1/\beta2/\gamma1$, 65% $\alpha2\beta2/\gamma1$, and 20% $\alpha2/\beta2/\gamma3$. The three heterotrimers are differentially regulated and have distinctive effects [320]. The $\alpha2/\beta2/\gamma3$ is quickly activated by physical work, whereas the other two aren't activated until (much) later. The

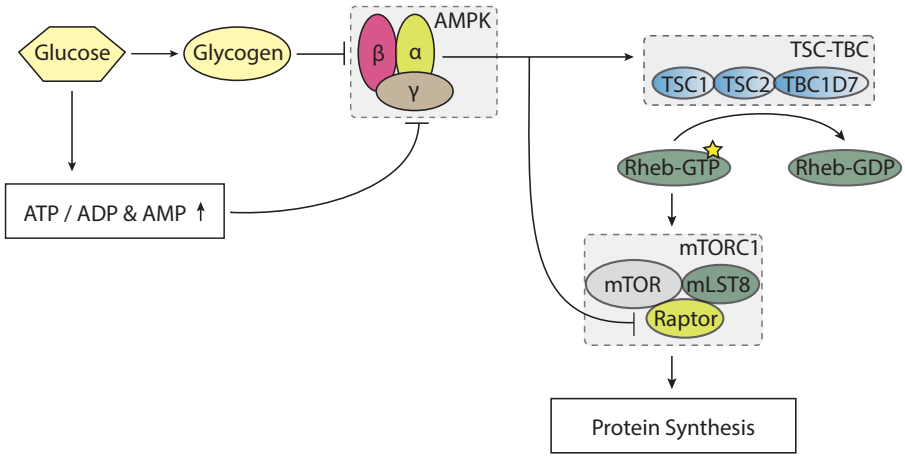


Figure 4.2: AMPK regulation by energy status. The γ-subunit interacts with the nucleotides ATP, ADP, and AMP. A high ATP to ADP/AMP ratio inhibits AMPK and a decrease in this ratio activates the kinase. Interaction of glycogen with the β-subunit leads to allosteric inhibition of AMPK. Activated AMPK phosphorylates TSC2 on two residues (Thr1227 and Ser1345) that are important for activation of the TSC-TBC complex. Additionally, activated AMPK phosphorylates the protein raptor on two residues (Ser722 and Ser792), which inhibits mTORC1 activity. Figure taken from Bond [59]. Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; mLST8, mammalian lethal with SEC13 protein 8; mTOR, mechanistic target of rapamycin; TSC1/2, tuberous sclerosis 1/2; TBC1D7, Tre2-Bub2-Cdc16 1 domain family member 7.

α1-containing isoform appears to inhibit muscle growth, while the α2-containing isoforms don't appear to do so [319].

The inhibition of muscle growth by AMPK occurs, at least in part, via inhibition of mTORC1. AMPK phosphorylates two residues (Thr1227 and Ser1345) on TSC2 that are important for its activation [235]. As described in Section 4.2.1, TSC2 inhibits mTORC1 activity, thus AMPK inhibits mTORC1 through activation of TSC2. In addition, raptor, a protein that is part of the mTORC1 complex, is also a substrate for AMPK [189]. Phosphorylation of this protein (on Ser722 and Ser792) also leads to inhibition of the complex. The antagonistic effect of AMPK on mTORC1 is thus imposed by phosphorylation of both TSC2 and raptor.

4.2.3 Regulation by amino acids

In the last few years, researchers have clarified a lot about how amino acids stimulate muscle growth. Amino acids are of course super important for protein synthesis, as they are the literal building blocks of proteins. It should therefore not come as a surprise that evolution has led to a tight regulation of protein synthesis based on the building blocks of this process.

The regulation of mTORC1 by amino acids primarily affects the cellular location of mTORC1. When a cell is amino acid deficient, mTORC1 is spread throughout the cytoplasm [398]. Addition of amino acids causes the complex to translocate to the lysosomal membrane [397], the site where mTORC1 is activated by Rheb-GTP (see also Section 4.2.1). The presence of amino acids thus ensures that the complex ends up in the right place in the cell to be activated.

Several mechanisms play a role in the translocation of mTORC1 to the lysosomal membrane. This translocation is established by so-called Ras-related GTPases. Rags, in turn, associate with a protein complex named Ragulator, which is anchored to the lysosomal membrane. The interaction of Rags with mTORC1 is dependent on their guanine nucleotide binding state, i.e., being bound to GDP or GTP.

There are four Rag proteins (RagA, RagB, RagC and RagD). RagA and RagB (RagA/B) bind to RagC and RagD (RagC/D) to form pairs of heterodimers. In a low amino acid availability state, RagA/B are bound to GDP and RagC/D are bound to GTP. In a high amino acid availability state, RagA/B are bound to GTP and RagC/D are bound to GDP and thus recruit mTORC1 to the lysosomal membrane. Amino acids regulate the guanine nucleotide binding state of the Rag proteins. This then causes mTORC1 to translocate to the lysosomal membrane for activation.

The Ragulator complex plays an important role in this. It associates with the Rag proteins, causing them to localize to the lysosomal membrane. In addition, the complex acts as a so-called guanine nucleotide exchange factor (GEF) for RagA/B [34]. This means that it can exchange the GDP of RagA/B for GTP, forming the active form of RagA/B.

In turn, the GEF activity of Ragulator is regulated by v-ATPase [34]. v-ATPase is a proton pomp present in the lysosomal membrane that pumps protons into the lysosome at the expense of ATP. This ensures the acidic environment of the lysosome is maintained. Ragulator associates with v-ATPase and amino acids cause a conformational change to the protein that consequently affects Ragulator to activate its GEF activity. How v-ATPase is regulated by amino acids is as yet unclear, but the signal appears to come from within the lysosome as a result of amino acid accumulation in its lumen [507].

In summary, the Ragulator complex, which associates with the lysosomal membrane, allows Rag proteins to interact with it and therefore enabling them to also reside at the lysosomal membrane. In addition, Ragulator can activate the RagA/B proteins, because amino acids stimulate the GEF activity of Ragulator via v-ATPase. This leads to translocation of mTORC1 to the lysosomal membrane, where it can be activated.

Whereas Ragulator acts as a GEF for RagA/B, another protein acts as a GAP for these proteins. The so-called GATOR1 complex exerts GAP activity towards RagA/B [33] and thus exchanges GTP for GDP, which leads to deactivation. GATOR1 thus has an inhibitory effect on mTORC1.

Another protein complex, called GATOR2, has an inhibitory effect on GATOR1 [33], as a result of which it has a stimulating effect on mTORC1. How GATOR2 manages to inhibit GATOR1 is unclear. The inhibitory effect of GATOR2 on GATOR1 is stimulated by amino acids, an effect that is mediated by so-called Sestrin proteins [89]. At the time, little is known about how these regulate GATOR2 either.

Finally, amino acids regulate the guanine nucleotide binding state of RagC/D. The enzyme that loads tRNA with leucine (leucyl-tRNA synthetase) shows GAP activity towards RagD in a leucine-dependent manner [191], although a later study failed to replicate these results [451]. What this later study did show was that the folliculin complex

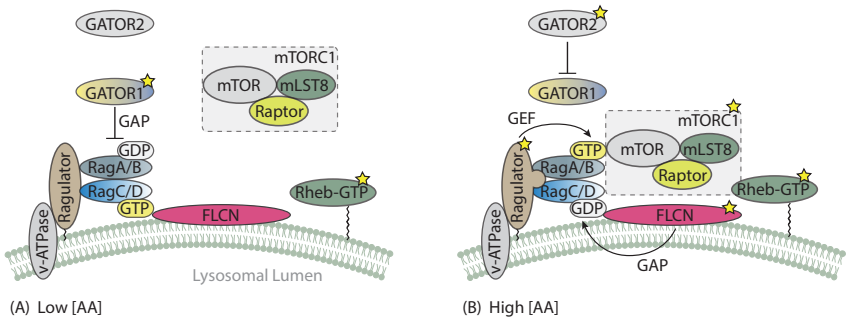


Figure 4.3: mTORC1 regulation by amino acids. *(A)* The Rag proteins are in their inactivate state at a low amino acid concentration and therefore cannot recruit mTORC1 to the lysosomal membrane for its activation by Rheb-GTP. Ragulator and v-ATPase are also in an inactive state, while GATOR1 exerts GAP activity towards RagA/B, keeping them inactive. *(B)* An increase in the amino acid concentration causes a conformational change in v-ATPase and Ragulator, which as a consequence initiates GEF activity towards RagA/B by Ragulator. FLCN and the proteins associated with it exert GAP activity towards RagC/D, which renders them active. Additionally, the GAP activity of GATOR1 is being inhibited by GATOR2. Together these actions lead to the formation of the active Rag heterodimers: GTP-bound RagA/B and GDP-bound RagC/D. These in turn recruit mTORC1 to the surface of the lysosomal membrane, allowing it to be activated by Rheb-GTP. Figure taken from Bond [59]. Abbreviations: FLCN, folliculin; GATOR1/2, GAP activity towards Rags 1/2; GDP, guanosine diphosphate; GTP, guanosine triphosphate; mLST8, mammalian lethal with SEC13 protein 8; mTORC1, mechanistic target of rapamycin complex 1.

exerts GAP activity towards RagC/D, which ultimately promotes mTORC1 activity.

Another mechanism by which leucyl-tRNA synthetase would activate mTORC1 is via the lipid kinase Vps34 [492]. In a leucine-dependent manner, the tRNA synthethase interacts with Vps34, rendering the latter active. Activated Vps34 then produces phosphatidylinositol 3-phosphate (PI(3)P). Subsequently, an interaction takes place between the formed PI(3)P and the PX domain of the phospholipase PLD1, causing it to translocate to the lysosome. PLD1 hydrolyses phosphatidylcholine to phosphatidic acid which is then able to activate mTORC1 directly. The regulation of mTORC1 by phosphatidic acid is discussed in more detail in the next section.

4.2.4 Regulation by mechanical stimuli

Given the important role of mTOR signaling in the regulation of protein synthesis and muscle volume, it's obvious that mechanical stimuli, such as by resistance exercise, also affect it. To date, two main mechanisms have been identified that regulate mTORC1 by mechanical stimuli.

One of these mechanisms, as is the case with the PI3K-Akt pathway, is the dissociation of TSC2 from the lysosomal membrane [237]. Mechanical load causes phosphorylation of TSC2 which makes it dissociate from the lysosomal membrane and prevents it from exerting GAP activity towards Rheb-GTP proteins, which are directly responsible for

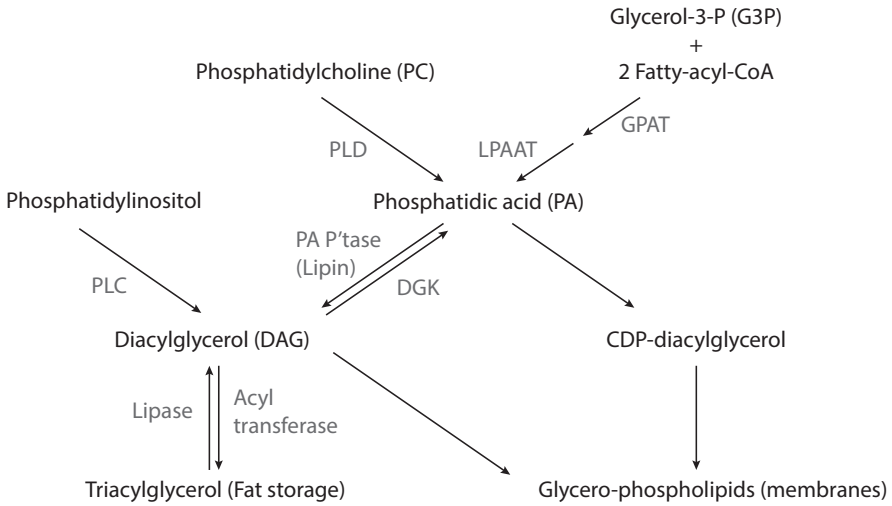


Figure 4.4: Phosphatidic acid (PA) can be synthesized from glycerol-3-phosphate (G3P), phosphatidylcholine (PC) and diacylglycerol (DAG). G3P is acetylated twice to form PA. First, acetylation takes place by GPAT, followed by LPAAT. PC is hydrolyzed by PLD to yield PA and DAG is phosphorylated by DGK to yield PA. DAG is derived from triacylglycerol and phosphatidylinositol. PA phosphatase is responsible for the dephosphorylation of PA to DAG. Several CDP diacylglycerol synthases produce CDP diacylglycerol from PA. Figure taken from Bond [59].

mTORC1 activation. In addition, mechanical stimuli promote the association of mTORC1 with the lysosomal membrane, further promoting its activation. It's uncertain what the underlying mechanism is.

Besides the Rheb-GTP proteins that can activate mTORC1, another molecule, phosphatidic acid, appears to be able to do this as well [493]. Phosphatidic acid is a diacylglycerol phospholipid. The stimulating effect of phosphatidic acid on mTORC1 mainly relies on two mechanisms of action:

1. displacement of the endogenous mTORC1 inhibitor FKBP38 from the FRB domain on mTORC1 (competitive inhibition);
2. allosteric stimulation of the kinase.

Box 4.3

i

Based on the findings of mechanistic studies, which demonstrated that phosphatidic acid plays an important role in mTORC1 activation, a clinical study was conducted in which phosphatidic acid was supplemented by resistance-trained men [217]. The study was performed in a double-blind and placebo-controlled fashion. The men that supplemented phosphatidic acid saw a greater increase in lean body mass than the placebo group. However, the result was not statistically significant ($P = 0.065$). Later, a few more studies with a similar design have investigated the effect of the supplement on muscle strength and mass. Phosphatidic acid supplementation is further discussed in

Chapter 10.

The amount of phosphatidic acid present in a cell is regulated by five different classes of enzymes [153]. Three of these are responsible for the synthesis of phosphatidic acid and two for its degradation. The balance between the two ultimately determines whether there will be an increase or a decrease in the phosphatidic acid concentration.

Glycerol-3-phosphate, phosphatidylcholine (PC) and diacylglycerol (DAG) are the precursors for the biosynthesis of phosphatidic acid. Glycerol-3-phosphate is acetylated twice to form phosphatidic acid. The first acetylation is catalyzed by the enzyme glycerol-3-phosphate acyltransferase (GPAT). The second is catalyzed by lysophosphatidic acid acyltransferase (LPAAT).

The formation of phosphatidic acid from phosphatidylcholine (PC) takes place by a hydrolysis reaction catalyzed by phospholipase D (PLD). It has long been believed that this reaction, catalyzed by PLD, was crucial in the mechanical stimulation-mediated rise in phosphatidic acid. The evidence was mainly based on the fact that the PLD inhibitor 1-butanol blocked mTORC1 activation in various experiments [224]. However, in 2009 it came to light that not all of 1-butanol's biological activity could be attributed to PLD inhibition. Moreover, it had already been shown that the degree of PLD activity as a result of mechanical stimuli correlates poorly over time with the measured increase in phosphatidic acid [223]. The PLD activity increases only briefly (measured after fifteen minutes) and then falls, whereas the phosphatidic acid concentration continues to rise after fifteen minutes.

Finally, phosphatidic acid is formed from diacylglycerol. Diacylglycerol is phosphorylated by a diacylglycerol kinase (DGK). There are many different isoforms of DGK and it has recently been demonstrated that the ζ-isoform is probably primarily responsible for the mechanical stimuli-mediated rise of phosphatidic acid in muscle cells [495]. In addition, the researchers demonstrated that PLD activity is not required for the rise of phosphatidic acid as a result of mechanical stimuli.

The regulation of the enzymes responsible for the breakdown of phosphatidic acid by mechanical stress is poorly understood.

II Supplements

5. Beta-alanine

5.1 Introduction

Beta-alanine is a fairly new dietary supplement that first grabbed the attention of athletes in 2006. In 2006, Roger C. Harris and his colleagues demonstrated that beta-alanine supplementation increased the carnosine concentration in muscle tissue [197]. The same Roger C. Harris was also the first to demonstrate that creatine supplementation increased the total amount of creatine in muscle tissue back in 1992 (see Chapter 8). Carnosine is a dipeptide of the two amino acids beta-alanine and L-histidine. Carnosine functions as an important pH buffer in muscle cells, as it is able to buffer protons (H^+). This can mainly be attributed to its imidazole group, located on the histidine residue [3]. The ergogenic effect of beta-alanine supplementation is also mainly based on an improvement of the pH buffering capacity. The beta-alanine availability is the limiting factor for the synthesis of carnosine, thus making its supplementation interesting. Carnosine itself could be supplemented as well, since it's broken down to beta-alanine and L-histidine by carnosinases. However, carnosine is more expensive to produce which makes it less cost effective. Carnosine can be found in a fairly high concentration in muscle cells, ranging from 10 to 40 mmol per kg of lean muscle mass [448]. This is comparable to the concentration of creatine phosphate

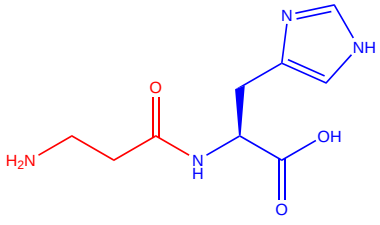


Figure 5.1: Structural formula of carnosine. Shown in red is the beta-alanine residue and in blue is the L-histidine residue. The imidazole group has a pKa value of 6.83.

found in muscle cells.

5.1.1 Biosynthesis

Beta-alanine, together with L-histidine, forms the precursor for the dipeptide carnosine. Since the effect of beta-alanine relies on its subsequent incorporation in carnosine, this section mainly deals with the biosynthesis of carnosine. The enzyme carnosine synthase is responsible for this (see Figure 5.2). The two substrates for this enzyme are beta-alanine and L-histidine. The enzyme also consumes an ATP molecule to catalyze the reaction [131]. The K_M values of carnosine synthase for its two substrates are estimated to be 16 μM and 1.8 mM for L-histidine [222] and beta-alanine [260], respectively. The availability of beta-alanine is thus the limiting factor for carnosine synthesis.

Supplementation of beta-alanine or carnosine to mice results in an increased expression of carnosine synthase [314]. Beta-alanine can be produced *de novo* by degradation of uracil [300]. Normally, however, most beta-alanine comes from the diet in the form of carnosine. Carnosine is mainly found in meat and fish and the average daily intake from these sources is estimated to bea round 0.3 g [29]. Supplementation of beta-alanine promotes carnosine synthesis in muscle cells, as demonstrated in 2006 by Roger C. Harris and his colleagues [197]. In this study, subjects were given 6.4 g of beta-alanine daily for 4 weeks, which lead to an increase in the intramuscular carnosine concentration of approximately 65 %.

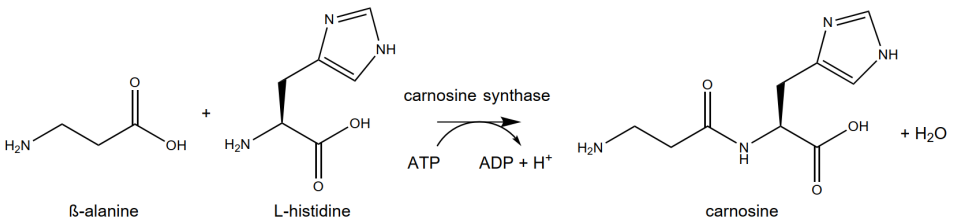


Figure 5.2: The carnosine synthesis. Beta-alanine and L-histidine are the two substrates for the enzyme carnosine synthase, which forms carnosine. The reaction is energized by hydrolysis of an ATP molecule.

5.1.2 Absorption

Beta-alanine is found in the diet in the form of carnosine. It's mainly found in meat and fish, as mentioned earlier. As a consequence, vegetarians have a lower amount of carnosine in their muscles than omnivores [146]. After ingestion of a carnosine source by mouth, it enters the gastrointestinal tract. Carnosine is absorbed from the lumen of the intestine by enterocytes that line the intestinal wall. This transport is probably mediated by the protein PEPT1, which enables carnosine to cross the brush border at the apical side of the enterocytes, together with a proton [421]. Part of the carnosine is then already hydrolyzed in the enterocytes. Research in SKPT cells (kidney cells) shows a low efflux of carnosine on the basolateral side [240]. If this is also the case *in vivo* with enterocytes, then only a small part of the carnosine absorbed by intestine ends up intact in the circulation. The carnosine is broken down by enzymes in the enterocytes to L-histidine and beta-alanine.

This degradation is catalyzed by carnosinases [39]. It is unknown whether paracellular transport of carnosine also plays a significant role in its uptake from the intestinal lumen. The remaining carnosine enters the circulation after it has been transported across the basolateral membrane. There are also carnosinases present in the circulation, which will further breakdown the carnosine that has made it intact.

In an experiment in which subjects were given 200 g of ground beef (approximately 248 mg carnosine), a peak concentration of 32.7 mg carnosine per liter of blood plasma was measured after 3.5 hours [344]. After 5.5 hours, no carnosine was detectable anymore. An average person has about 5 liters of blood flowing through their veins, and about 55 % of it is occupied by the blood plasma. This amounts to a total of roughly 2.5 liters of blood plasma. This means that there was about 100 mg carnosine in the circulation of the subjects when the peak concentration was measured. These data lead to the conclusion that a large part of the carnosine still reaches the bloodstream intact after absorption by the gastrointestinal tract. However, to be useful for increasing the carnosine concentration in muscle cells, it still needs to be hydrolyzed. The reason for this is that the uptake of intact carnosine by muscle cells is very low [37].

The absorption of beta-alanine is thus crucial to increase the carnosine concentration in muscle cells. Beta-alanine is absorbed at the apical side of enterocytes by two transporters [66]. A high affinity, low capacity transporter that is Na^+- and Cl^--dependent, and a low affinity, high capacity transporter that is proton dependent. The latter also facilitates the transport of other amino acids and derivatives thereof, including trimethylglycine. Once beta-alanine enters the circulation, it can be taken up by the muscle cells so that carnosine synthase can form carnosine from it with L-histidine. The uptake by muscle cells is probably also facilitated by Na^+- and Cl^--dependent transport [30]. Recently, researchers confirmed the presence of the Na^+ and Cl^--dependent transporter TauT (taurine transporter) in human m. gastrocnemius [145]. They also demonstrated the presence of the transporter PAT1 (proton-coupled amino acid transporter 1), a proton-coupled amino acid transporter. TauT mRNA expression was also found to be increased after eight weeks of beta-alanine supplementation in mice [145].

The loading of carnosine into muscle tissue seems to follow a linear dose-response relationship [429]. This means that the total amount of beta-alanine ingested is the primary determinant of the increase in carnosine concentration in muscle cells. Remarkably, the increase in this concentration is much lower than you would expect based on the amount of beta-alanine ingested. One study calculated that only about 2.8 % of the ingested beta-alanine ended up being incorporated into muscle tissue as carnosine. As such, it's worth the effort to seek for strategies to improve the incorporation of ingested beta-alanine into muscle as carnosine.

The researchers of that same study hypothesized that, similar to creatine, insulin could improve absorption. After all, the beta-alanine transporter TauT is Na^+-dependent and insulin stimulates the sodium potassium pump activity, probably because insulin leads to translocation of sodium potassium pumps to the sarcolemma [432]. This, in turn, stimulates the secondary transport of sodium dependent transporters, such as the TauT. The researchers have put this hypothesis to the test by giving subjects beta-alanine together with two meals, so that the increase in beta-alanine in the circulation coincided with an increase in the serum insulin concentration. Indeed, coingestion with a meal led to a greater increase in the intramuscular carnosine concentration in the soleus muscle, but not in the gastrocnemius muscle. This is believed to be because the insulin-mediated translocation

of sodium potassium pumps to the sarcolemma appears to occur only in oxidative muscle fibers, and not glycolytic fibers [275]. The soleus muscle in particular has many oxidative muscle fibers [171]. Previous research in rats also showed that the muscles consisting of a large portion of oxidative muscle fibers in particular were more sensitive to the effects of insulin [239]. Nevertheless, it should be noted that the gastrocnemius also seems to consist to a great extent of oxidative muscle fibers [110]. Thus, it's not very clear what actually caused this difference.

In addition to combining it with an insulinogenic meal, there is also evidence that exercising the muscle itself further increases the carnosine storage after beta-alanine ingestion [41]. Combining a workout with a meal may work the best for this. For example, a storage efficiency of 2.39 % is noted without a meal or training, whereas it was as high as 5.65 to 5.82 % when combined with a meal and exercise training [52].

5.1.3 Metabolism and excretion

The retention of beta-alanine is quite high (97–98 %), but only a fraction of this is stored as carnosine in muscle tissue [428, 52]. It's possible that tissues other than muscle tissue also store some of the ingested beta-alanine as carnosine. However, the absolute amount of carnosine in other tissues is considerably lower [152]. The amount in muscle tissue is much greater than in any other tissue. For example, muscle tissue contains 5–8 mmol carnosine per kg [124]. The molar mass of carnosine is 226.23 g/mol. Assuming a 70 kg person whose muscle mass comprises 40 % of total body weight (28 kg), 31.7–50.7 g of carnosine would be stored in the muscle mass. In comparison, the kidney contains about 61 mcg carnosine per g [152]. Assuming a weight of 150 g per kidney, the total amount of carnosine in the kidneys is only 0.02 g. Similar amounts are found in the heart, stomach, and jejunum [152]. Mainly because of this, it's likely that most of beta-alanine that is not stored in muscle tissue is metabolized and not stored elsewhere in the body.

It's believed that a large portion of the ingested beta-alanine simply gets oxidized [428]. Indeed, studies in rats have demonstrated that more than 70 % of the administered beta-alanine was broken down to carbon dioxide, and part was also incorporated into acetyl-containing substances such as fatty acids and cholesterol [181]. To function as a substrate for the citric acid cycle (see Section 2.4.1), beta-alanine needs to be stripped off its amino group. This is done by the enzyme GABA transaminase, which catalyzes the transamination reaction [181, 42]. The mRNA expression of the enzyme is also upregulated in muscle cells by beta-alanine supplementation [145]. The product of this transamination reaction is malonic acid semialdehyde which can be further metabolized to acetyl-CoA or acetaldehyde [181].

The breakdown of intramuscular carnosine proceeds very slowly. After beta-alanine supplementation is stopped, the carnosine concentration decreases by 2–4 % per week [29, 429]. Therefore, after an approximately 30 to 45 % increase in intramuscular carnosine concentration, it's estimated that a washout period of 15 to 20 weeks is required to perform a reliable crossover designed clinical trial [429].

5.2 Mechanism of action

Ever since the discovery of carnosine, research into its physiological role has focused on muscle tissue. Nevertheless, to this day it's still not known what the main role of carnosine in muscle tissue is. The most obvious role of carnosine in muscle tissue can be ascribed to

its imidazole group. This imidazole group has a pKa value of 6.83. This makes it ideal to act as a pH buffer and thus to hold off an increase in the concentration of protons. Another role of carnosine appears to lie in regulation of the excitation-contraction coupling that initiates muscle contraction (see Section 1.3). This has to do with the release of Ca^{2+} ions from the sarcoplasmic reticulum (SR). The released Ca^{2+} will bind to troponin C, allowing myosin to form cross bridges with actin and thus to let the muscle contract. Recently, a hypothesis has been proposed that unites the regulation of the H^+ and Ca^{2+} ions by carnosine in the cell [434].

5.2.1 pH buffer

A lot of ATP is hydrolyzed by the myosin ATPases to make the sarcomeres of the muscle contract during intense exercise (see also Chapter 2, equation 2.1). A product of this reaction is a proton (H^+). During a low rate of energy expenditure, (almost) all protons resulting from ATP hydrolysis are used in the mitochondria for the respiratory chain. However, with a higher rate of energy expenditure, in which the ATP turnover in the cytosol exceeds the production of ATP in the mitochondria, these protons can accumulate and the muscle acidifies as a result. Part of these protons are consumed during lactate production, after all, this reaction consumes a proton (see Section 2.3.1). Part of it is also buffered by the produced P_i. Although P_i and H^+ are produced in a 1:1 ratio by ATP hydrolysis along with ADP, only ADP and P_i are used as substrates for glycolysis, but not H^+ (see Chapter 2, equation 2.3). As a result, a surplus of H^+ develops that cannot be buffered by the P_i produced by the same reaction. In addition, one proton is generated when glycogen is used to feed glycolysis, and two protons when glucose is used directly as a substrate [384]. As a result, the pH value in the muscle cells will drop.

Beta-alanine has an imidazole group with a pKa value of approximately 6.8, which allows it to function as a proton buffer in the muscle cells. It's generally believed that a drop in the pH level in a muscle cell decreases performance. However, the negative effect of exercise-induced acidification on the contractile properties appears to be pretty minor [10]. Nonetheless, dietary supplements that act on the drop in a muscle cell's pH level as a result of exercise have received a lot of attention from researchers, including beta-alanine. A clinical study demonstrated a positive correlation between the intramuscular carnosine concentration and the *in vitro* buffer capacity [28]. In this study, a muscle biopsy was taken and then homogenized. Then it was examined how many protons were needed to decrease the pH value from 7.1 to 6.5. However, no beta-alanine was supplemented in this study. In another study they did supplement beta-alanine [183]. Nevertheless, the researchers didn't find a difference between the group receiving a placebo and the group receiving beta-alanine in their *in vitro* buffer capacity. It's estimated that *in vivo* the buffer capacity of carnosine is about 7 % of the total buffering capacity [295]. All in all, the pH buffering capacity itself appears to be quite modest.

5.2.2 Increased Ca^{2+} sensitivity

As covered in sections 1.2 and 1.3, an increase in the Ca^{2+} concentration in muscle cells initiates muscle contraction. The calcium ions bind to troponin C, freeing binding sites on actin and thus making them available for the myosin heads to form cross bridges. The higher the Ca^{2+} concentration, the more cross bridges that can be formed. The Ca^{2+} concentration thus ultimately determines the force produced by a muscle cell, since the number of cross bridges dictates the force of contraction. When the Ca^{2+} concentration is

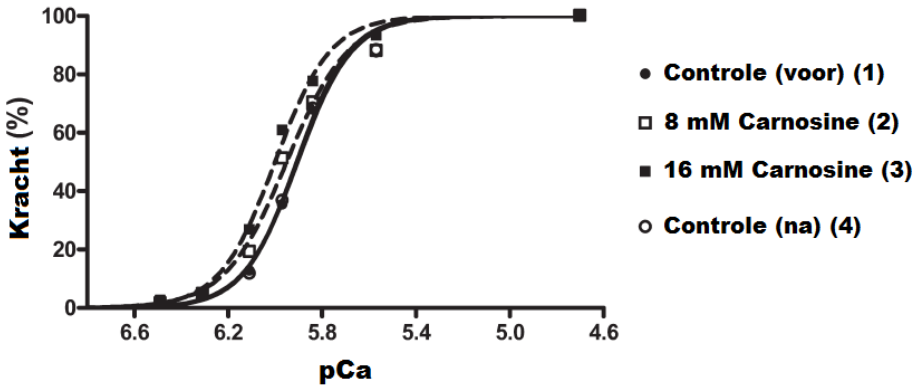


Figure 5.3: The force-pCa curve of human muscle fibers after addition of carnosine at two different concentrations (8 mM and 16 mM). The Ca^{2+} sensitivity of the contractile apparatus is enhanced after carnosine addition, because the curve is shifted to the left. Analogous to the usage of pH as a measure of the H^+ concentration, pCa expresses the intracellular Ca^{2+} concentration as a negative logarithm of the Ca^{2+} concentration in mol/L. Figure adapted from Dutka et al. [136].

plotted against the relative contraction force, a logarithmic curve is formed. The threshold for contraction lies around 10^{-7} mol/L, and maximum contraction force is reached at a concentration of about 10^{-4} mol/L. Research in human type I and type II fibers suggests that beta-alanine supplementation may shift this curve to the left [136]. This means that with the same Ca^{2+} concentration more cross bridges are formed. For example, a Ca^{2+} concentration that results in 50 % of maximum contraction force, will result in 60 % of maximum contraction when the carnosine concentration is higher. Thus, with the same Ca^{2+} concentration, more power is delivered during submaximal exertions. This could potentially make an athlete less likely to lose performance with such efforts. The increased Ca^{2+} sensitivity of the contractile apparatus at higher carnosine concentrations might be attributed to the role of carnosine as a shuttle system. This role is discussed in Section 5.2.3.

5.2.3 Carnosine shuttle hypothesis

Recently, a hypothesis has been proposed that unites the role of carnosine as a pH buffer and a Ca^{2+} regulator. Since both protons and calcium ions can bind to carnosine, Swietach et al. postulated that carnosine acts as a diffusible Ca^{2+}/H^+ exchanger [434, 433]. Both ions are hardly present in their free form in the cytoplasm. As such, they are dependent on carrier molecules for their diffusion. During contraction, protons are released from the ATP hydrolysis carried out by the ATPases of the myosin heads. This creates a large concentration of protons in close vicinity of the sarcomeres. This higher proton concentration (and therefore locally lower pH value) promotes the protonation of molecules, including carnosine. This causes a discharge of calcium ions, which also bind to carnosine. On the other hand, the action potential that triggers muscle contraction initiates an efflux of calcium ions from the sarcoplasmic reticulum (SR). This sharp rise in Ca^{2+} concentration

leads to the opposite process: the 'loading' of carnosine with calcium ions, and the discharge of the protons. In summary, carnosine, as a small dipeptide, functions as a mobile buffer for these molecules in the cell, allowing a rapid exchange of them between the cellular compartments. This may be an important mechanism responsible for the ergogenic effects of carnosine loading in the muscles [52].

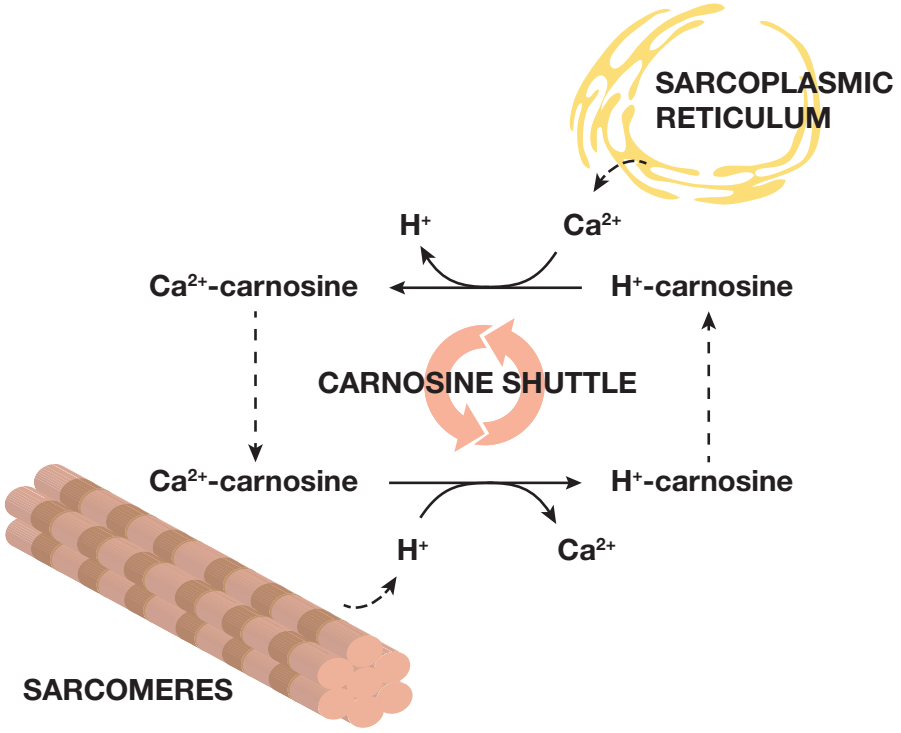


Figure 5.4: The carnosine shuttle hypothesis. Carnosine can act as a diffusible Ca^{2+}/H^{+} exchanger between the sarcomeres and the sarcoplasmic reticulum. It carries calcium ions to the sarcomeres, and protons away from them. Figure based on Blancquaert et al. [52].

5.2.4 Antioxidant activity

During muscle contraction more reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated than at rest [363]. These are molecules that are highly reactive, causing them to react with other molecules present in a cell, such as lipids, proteins, carbohydrates, and nucleic acids, thus causing them damage. Consequently, these reactive compounds are at the forefront of various pathologies. Nevertheless, they also play an important role in the normal functioning of many vital processes [65]. In addition, they play an important role in muscle fatigue.

The reactive compounds can be derived from the respiratory chain and various other enzyme-catalyzed reactions involved in cell metabolism, as well as from radiation. Nonethe-

less, the primary source of intracellular ROS in most cells is the mitochondria, from the respiratory chain [31]. This predominately leads to the formation of superoxide ($O_2^{\cdot -}$) due to leakage of electrons from the respiratory chain which interact with O_2. The reaction is catalyzed by NADPH oxidase (NOX) and NADPH functions as the reducing agent. Although superoxide itself isn't a particularly powerful oxidant, it's the precursor for many other ROS and is therefore involved in oxidative chain reactions [452]. However, it should be noted that there doesn't appear to be a fixed relationship between mitochondrial oxygen consumption and $O_2^{\cdot -}$ production. As such, it's wrong to assume that its production increases in the mitochondria when there's an increase in oxygen consumption due to muscle contraction [90]. The main source of $O_2^{\cdot -}$ in muscle cells are probably NOX enzymes [395]. As said, these enzymes catalyze the reduction of molecular oxygen to superoxide with NADPH as a reducing agent. The reaction catalyzed by NOX is shown in Reaction 5.1:

$$\mathrm{NADPH} + 2\,\mathrm{O_2} \rightarrow \mathrm{NADP^+} + \mathrm{O_2^{\cdot -}} + \mathrm{H^+}. \tag{5.1}$$

The NOX enzymes can be found in different places in a muscle cell. In addition to the mitochondria, they also occur in the sarcolemma, the T-tubules, and the sarcoplasmic reticulum. This means that superoxide generated by NOX enzymes is also located outside the mitochondria, and therefore has a different effect on the cell than superoxide produced in the mitochondria. This is an important point to consider, given that the subcellular location of ROS is important for its positive or negative effect on muscles. Although it was previously believed that any rise in ROS or RNS was harmful to cells and was involved in muscle fatigue, there is now more than enough evidence that indicates that this assumption is too simplistic. Indeed, increases in ROS or RNS also play an important role in adaptation of muscle cells to exercise, and in some cases antioxidant supplementation has been shown to exert a negative effect on adaptation to high-intensity exercise [310]. Nevertheless, it's suggested that the antioxidant action of carnosine [261] may be beneficial [448]. However, it has never been directly researched whether this plays a role in the ergogenic effect of beta-alanine. Such research is certainly necessary, given the complex role that ROS and RNS have in muscle cells, as discussed.

5.3 Clinical results

Only a small number of clinical studies have looked at the effect of beta-alanine on body composition or strength performance. In view of the purported role as a pH buffer, there has been keen interest in the effect of this on sports where acidification can be limiting, such as rowing, sprinting, cycling and other sports that often involve intense exercise lasting longer than a minute at a time. A meta-analysis published in 2012 concluded that beta-alanine supplementation has a positive effect on exercise performance when exercise lasts longer than 60 seconds [213]. A more recent meta-analysis published in 2016 concludes that beta-alanine is especially effective in exercise lasting 0.5 to 10 minutes [403]. Shorter lasting exercise bouts clearly don't demonstrate any benefit.

Studies in which beta-alanine supplementation was combined with resistance exercise are limited. A first study published in 2006 investigated the effect of beta-alanine supplementation in combination with creatine (CA) compared to creatine alone (C) or a placebo (P) [214]. Subjects had at least two years of resistance exercise experience and

during the 10-week intervention they trained four times a week. The 1-RM (1-repetition maximum) bench press and squat were used as a measure of strength. Body composition was determined by a dual-energy X-ray absorptiometry (DXA) scan. A significant decrease in body fat percentage and increase in lean body mass (LBM) was found in the CA group compared to the P group. Both the CA and C groups showed a significant increase in their 1-RM bench press and squat compared to the P group. Furthermore, squat and bench press volumes were significantly greater in the CA group compared to the P group. These results suggest that beta-alanine in combination with creatine may be able to aid in achieving a higher training volume. This higher training volume could then possibly translate into more muscle growth. A problem, however, is that the study lacked a group that only received beta-alanine.

Kendrick et al. also combined a 10-week resistance exercise program with beta-alanine supplementation [255]. In contrast to the earlier study by Hoffman et al., this time the supplement was not combined with creatine. The subjects were young Vietnamese physical education students. They were divided into a beta-alanine group that received 6.4 g daily, and a placebo group. A resistance exercise program was followed for ten weeks, with four workouts per week. After these ten weeks, body strength (determined by a 1-RM bench press, box squat and deadlift) had improved in both groups, but no significant group-$\times$-time interaction was found. The same was found for fatigue resistance and body composition. Body composition was determined in this study using a skin caliper. Muscle biopsies did confirm a significant increase in muscle carnosine concentration in the supplementation group compared to the placebo group. These results suggest that beta-alanine supplementation per se has no effect on strength, fatigue resistance, or body composition in beginners over a 10-week period.

Other research in college football players did report significantly lower subjective feelings of fatigue and a trend ($P = 0.09$) for a greater training volume in the beta-alanine group compared to the placebo group. Training status and total volume may play a role in this.

A study in collegiate wrestlers showed a surprising effect on body composition [256]. Most of the wrestlers who took part in the study tried to lose weight. A resistance training program was followed for a total of eight weeks, training three times a week. Fat was lost and LBM gained in both the placebo and beta-alanine groups. However, the increase in LBM was greatest in the beta-alanine group (1 kg vs 0.5 kg). Nevertheless, it should be noted that calorie intake was lower in the placebo group (21.9 kcal/kg bw) compared to the beta-alanine group (25.7 kcal/kg bw). This could explain the lower LBM gain in the placebo group. A study combining HIIT training with beta-alanine or placebo demonstrated a significant time interaction in LBM (determined with a BODPOD) in the beta-alanine group that wasn't found in the placebo group [418].

The positive effect on body composition that sometimes is seen in studies may be attributed to the increase in total training volume. This, in turn, might lead to an increase in muscle hypertrophy. An alternative explanation could be that it's due to the increased muscle carnosine concentration itself. The concentration increase is comparable to the concentration increase of creatine as a result of supplementation, and thus leads to an increased osmolality. As a consequence, water is being sucked into the muscle cells. (An explanation of osmosis is given in Box 11.1.)

5.4 Safety

There is currently too little data to state that long-term beta-alanine use is safe. Some short-term studies have been done, however. A study in which a small group of healthy men received 3.2 g beta-alanine daily for 4 weeks showed no significant changes in biochemical and haematological blood values compared to the placebo group [197]. Later research by Stellingwerff et al. confirmed these results [429]. The number of participants in this study was slightly higher ($n = 31$) and the subjects received beta-alanine for 8 weeks. In addition to the placebo group, there were two groups that received a slow-release formulation. One group of these received 3.2 g daily for the first 4 weeks and 1.6 g daily for the final 4 weeks, whereas the other group received 1.6 g daily from the start. Again, no abnormalities were found in biochemical and haematological blood values.

At present, the only reported side effect of beta-alanine use appears to be paresthesia. This basically feels like pins and needles on the skin or tingling, but is also sometimes described as a burning sensation. It's as yet unclear why beta-alanine causes this, but it has been hypothesized that beta-alanine binds to a G-protein coupled receptor located on neurons that innervate the skin [417, 448]. Given the pH buffering capacity of beta-alanine by subsequent formation of carnosine, it may also be possible that a short-lived alkalosis is triggered in these neurons. An alkalosis can also lead to paresthesia (such as with hyperventilation). However, none of these hypotheses has been directly tested, and the cause is therefore still speculative. The paresthesia is believed to be harmless. Spreading a beta-alanine dose over several smaller doses during the day could reduce paresthesia. Beta-alanine is also available as a slow-release supplement, which could help against paresthesia [117].

Finally, there is some concern about the inhibition of taurine uptake as a result of beta-alanine supplementation. Both taurine and beta-alanine are β-amino acids and share the same transporter. This could lead to competitive inhibition of taurine uptake due to an increase of beta-alanine in the circulation. In an animal study, beta-alanine was added to the drinking water of cats [287]. After twenty weeks, there was a decrease in the taurine concentration in the brain and damage to the cerebellum was observed. However, the amount of beta-alanine that the cats received was quite high, about 500 g over the course of those twenty weeks. In addition, taurine is an essential amino acid for cats, but not for humans. Nevertheless, beta-alanine, in large amounts, is able to induce taurine deficiencies in several animal models. In the clinical study by Harris et al., no significant increase in urine taurine loss was measured despite an increased plasma taurine concentration [197]. Additionally, another trial in cyclists who received beta-alanine for ten weeks, no change was found in the taurine concentration in muscle cells [210]. Symptoms of a taurine deficiency, such as muscle cramps, have also not been reported in clinical trials to date. However, clinical research into the effect of long-term beta-alanine use on taurine levels is limited. Moreover, data is also lacking on, for example, the taurine concentration in other tissues, such as heart tissue, with beta-alanine use. Therefore, due to a lack of data, caution appears to be warranted with long-term supplementation.

5.5 Conclusion

Beta-alanine is able to significantly increase the carnosine concentration in muscles. This way it improves the pH buffering capacity of the muscles. Because of this, the supplement appears to be especially effective for exercise where acidification can be limiting. It's

probably not effective for gaining muscle strength. However, its use may allow an increase in training volume. There are also some indications that supplementation improves body composition. The mechanism for this is uncertain.

A common dosage is around 3–6 g daily. The amount that is effectively stored in the muscle cells as carnosine is quite low. Efficacy might be improved by combining the intake with a (insulinogenic) meal. Exercising the muscles in itself may also improve the storage of beta-alanine as carnosine.

Many people experience paresthesia (pins and needles sensation) when using beta-alanine. This seems to be the only side effect of beta-alanine. Paresthesia can be prevented or reduced by spreading the dose over the course of the day, and by avoiding intake on an empty stomach. In addition, there is a commercially available form of beta-alanine in which it's more slowly released into the circulation, which could also help. However, long-term studies on the supplement's safety are lacking.

6. β-Hydroxy β-methylbutyric acid (HMB)

6.1 Introduction

β-Hydroxy β-methylbutyric acid (HMB) is a metabolite of the essential amino acid leucine. Leucine belongs to the branched-chain amino acids (BCAAs) and is considered an important amino acid for stimulating protein synthesis, as well as inhibiting protein degradation [129].

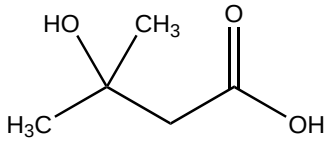


Figure 6.1: Structural formula of β-hydroxy β-methylbutyric acid (HMB). Under physiological conditions the carboxyl group (-COOH) is deprotonated.

In 1975, leucine was first put forward as an important regulator of protein turnover in muscle tissue [76]. In this research, it was discovered that extracellular leucine, but not isoleucine or valine (also BCAAs), increased protein synthesis and decreased protein degradation in the hemidiaphragm of rats.

Because of this role of leucine in amino acid metabolism, its metabolites have also been examined. Based on animal studies, Nissen et al. hypothesized that the leucine metabolite HMB was responsible for the inhibitory effect on protein degradation [331]. To test this hypothesis, the researchers performed two experiments in which HMB was given to men.

In the first experiment, subjects were randomly assigned to receive 0, 1.5, or 3.0 g HMB daily and 117 or 175 g protein daily. The subjects performed resistance exercise three times a week during the 3-week study period. Urinary excretion of 3-methylhistidine (3-MH) was used as a surrogate marker for muscle protein breakdown (see Box 6.1). After 1 week there was a trend ($P = 0.13$) towards a linear effect of HMB on inhibition of 3-MH excretion (this increased in all groups due to resistance exercise), and statistical

significance was reached after week 2. However, the effect disappeared after week 3. In addition, a statistically significant effect of HMB on muscle strength was observed relative to the control group.[1]

In the second experiment, resistance exercise was performed 6 days a week, and aerobic training at least three times a week. The study period was 7 weeks this time and the researchers looked at the effect of HMB supplementation on body composition in particular. This was determined by bioelectrical impedance analysis (BIA). A significant increase in lean body mass was observed in the HMB group from day 14 to day 39. [2]

Taken together, these data suggested that HMB was responsible, at least in part, for the observed effects of leucine. Since this pilot study, much clinical research has been conducted in order to examine the effects of HMB on sports performance and body composition.

Box 6.1

3-Methylhistidine (3-MH) is a methylated form of the amino acid histidine. 3-MH is abundantly present in the contractile muscle proteins actin and myosin. When these muscle proteins are broken down, 3-MH is released. 3-MH cannot be reused for protein synthesis and is simply excreted in the urine. This makes 3-MH an interesting amino acid to use as a surrogate marker of muscle protein breakdown. However, the use of 3-MH as a surrogate marker for muscle protein breakdown is not without a few caveats [381]. Naturally, 3-MH also occurs in tissues other than muscle tissue, such as the skin and the intestinal system. Moreover, it's also present in the diet.

6.1.1 Biosynthesis

HMB is formed from the amino acid leucine. The first step in HMB biosynthesis is the reversible transamination of leucine to its α-ketoacid α-ketoisocaproate (α-KIC). This reaction mainly takes place outside the liver and is catalyzed by the enzyme BCAA aminotransferase (BCAAT) [53]. BCAAT activity is highest in skeletal muscle, which is estimated to account for more than half of total BCAAT activity [68]. Additionally, significant BCAAT activity takes place in the brain and adipose tissue, while the activity in the liver is quite modest. As such, a lot of α-KIC is formed in the muscles.

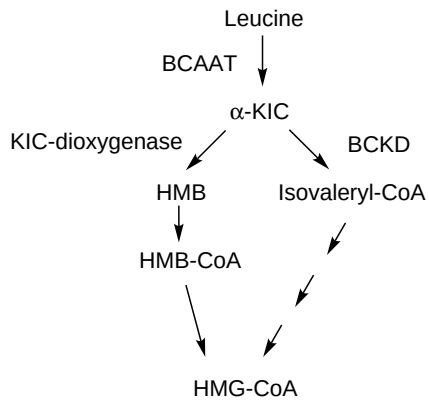


Figure 6.2: HMB biosynthesis.

α-KIC can then follow two different metabolic pathways. One of these forms HMB directly and takes place in the cytosol. In this, α-KIC is simply oxidized to HMB in the cytosol. This reaction is catalyzed by the enzyme KIC dioxygenase. The K_m value of this enzyme is estimated to be 120 μM [332]. The second pathway that α-KIC can follow starts with oxidation to isovaleryl-CoA in the mitochondria. This reaction is catalyzed by

[1] However, seven of the fifteen subjects in the HMB group also received a protein supplement. The subjects in the control group didn't receive this.

[2] The HMB group also received a protein supplement, whereas the placebo group didn't.

the enzyme branched-chain ketoacid dehydrogenase (BKCD), with an estimated K_m of 10–40 μM [332]. Subsequently, some reactions take place which ultimately result in the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).

It's estimated that about 5 % of the ingested leucine is metabolized to HMB [497]. Assuming a daily protein intake of 150 g, of which approximately 10 % (15 g) consists of leucine, this yields about 0.75 g of HMB daily.

6.1.2 Absorption

HMB is supplemented orally in the form of capsules or gels and is virtually non-existent in the diet in significant amounts. It's commercially available as a calcium salt (Ca^{2+}-HMB) and in free acid form (HMB-FA). It's absorbed by the body from the gastrointestinal tract and eventually ends up in the circulation. Since HMB is a polar molecule, it relies on a transporter to get across the cell membrane. However, to date no transporter for HMB has been identified.

Oral administration of 1 g Ca^{2+}-HMB gives a peak in the plasma concentration curve of about 115–130 μmol/L about 2 hours after ingestion [469, 155]. An intake of 3 g Ca^{2+}-HMB yields a peak in the plasma concentration of $\sim$480 μmol/L approximately 1 hour after ingestion. This peak after intake of 3 g Ca^{2+}-HMB is lower ($\sim$350 μmol/L) and is reached later (approximately 2 hours after intake) when combined with 75 g glucose. The area under the plasma concentration curve is significantly smaller the first 3 hours after ingestion when Ca^{2+}-HMB is combined with glucose. After these 3 hours, it still looks smaller, but this is not statistically significant ($P = 0.106$) [469]. The half-life is approximately 2.3–3.4 hours and is independent of the dose (1 or 3 g) [469, 155].

Administration of 1 g HMB-FA leads to a significantly faster peak in the plasma concentration curve [155]. This peak is reached about 0.5 hours after ingestion and is roughly twice as high compared to Ca^{2+}-HMB ingested as capsules. The area under the plasma concentration curve is also significantly larger the first 3 hours after ingestion. This shouldn't come as a surprise, given that the peak concentration is reached quite late after ingestion of Ca^{2+}-HMB capsules. The difference in the area under the plasma concentration curve is much smaller when looking at the first 24 hours instead of 3 hours after ingestion. The half-life appears to be slightly shorter for HMB-FA and the clearance is also higher. In combination with the fact that the 24-hour urinary excretion of the free acid gel form is not significantly different from the Ca^{2+}-HMB taken as capsules, these data suggest that the free acid form has a higher retention in the body.

The intake of HMB leads to a sharp increase in the intramuscular HMB concentration. This increases from 7 $\pm$3 μmol/L in a fasted state to 96 $\pm$13 μmol/L 2.5 hours after ingestion of 3.42 g HMB-FA [478]. In the same study, an equal dose of leucine was unable to affect the intramuscular HMB concentration.

6.1.3 Metabolism and excretion

The main metabolic pathway followed by HMB appears to be the conversion to HMG-CoA [332]. First, there is a transfer of HMB to coenzyme-A which forms HMB-CoA. After this, carboxylation can take place to form HMG-CoA. HMG-CoA can also be formed indirectly by dehydration of HMB-CoA to β-methylcrotonyl-CoA (MC-CoA), followed by a carboxylation reaction yielding β-methylgluconyl-CoA (MG-CoA) and finally hydration to HMG-CoA. HMG-CoA can then serve as a starting point for *de novo* cholesterol synthesis, in which HMG-CoA is reduced to mevalonate by HMG-CoA reductase. This

Study	Form	Dose (g)	C_{max} (μmol/L)	T_{max} (h)
Vukovich e.a. (2001)	Ca^{2+}-HMB	1	115	2
Vukovich e.a. (2001)	Ca^{2+}-HMB	3	480	1
Vukovich e.a. (2001)	Ca^{2+}-HMB + glucose	3 + 75	350	1.9
Fuller e.a. (2011)	Ca^{2+}-HMB	0.8	131	2
Fuller e.a. (2011)	HMB-FA	0.8	259	0.5
Wilkinson e.a. (2013)	HMB-FA	3.42	408	0.5

Table 6.1: Pharmacokinetic data of HMB.

is the flux determining step for *de novo* cholesterol synthesis. This step is also targeted by the group of drugs named statins. In a short-term (3 days) small-scale ($n = 8$) trial, a slight increase in serum cholesterol was found [230]. Two other longer-term trials (8 weeks and 28 days) showed no change in serum cholesterol [156, 267]. One small trial even demonstrated a decrease [332].

However, a significant portion of ingested HMB ends up unchanged in the urine. After a single administration of 3 g Ca^{2+}-HMB, nearly 30 % was excreted in the urine in the following 24 hours. After administration of 1 g of HMB (both as calcium salt and free acid form) approximately 15 % was excreted in the urine [155]. These results suggest that a higher percentage of the ingested HMB may end up in the urine with higher doses.

6.2 Mechanism of action

To date, several mechanisms of action have been postulated for HMB [8], of which the reciprocal interaction and relative contribution of each has not yet been elucidated. The following mechanisms could play a role in the effects of HMB supplementation:

1. increased protein synthesis through stimulation of mTOR signaling;
2. inhibition of protein degradation by the proteasome;
3. improved membrane integrity of the sarcolemma by stimulation of the mevalonate pathway;
4. stimulation of proliferation, differentiation and fusion of the satellite cells by the MAPK and PI3K pathways;
5. modulation of the autophagic-lysosomal system.

6.2.1 Stimulation of mTOR signaling

The mechanistic target of rapamycin (mTOR) pathway plays a governing role in the intracellular signaling of muscle growth, as discussed in detail in Section 4.2. Briefly: mTOR forms a protein complex called mTORC1 with other proteins. This protein complex integrates various signals, including for example amino acid availability, to become active. The complex stimulates protein synthesis once activated. The complex does this by phosphorylating proteins involved in protein synthesis, which affects their activity.

In an animal experiment with rats, HMB supplementation led to an increased phosphorylation state of one of these proteins in muscle tissue, namely S6K1 [356]. Another study also found an increase in phosphorylation of S6K1 by HMB in C2C12 myoblasts that were incubated with proteolysis-inducing factor (PIF, a protein that stimulates proteolysis and

inhibits protein synthesis) [142]. In addition, the amount of mTOR also increased sharply [356]. Remarkably, no increase in the phosphorylation state (on Ser473) of Akt was found (see Section 4.2.1. However, in differentiated C2C12 cells, an increase in the phosphorylation state of Akt was found ten and thirty minutes after incubation [259]. It could therefore be that this increase was missed in the other study, because the measurements were taken fifteen to eighteen hours after HMB supplementation. An increase in mTOR-P was also measured thirty minutes after incubation with HMB [259]. Administration of a PI3K inhibitor (PI3K is an upstream regulator of Akt, and thus mTOR) canceled this increase.

However, this increase in Akt phosphorylation wasn't seen in humans ingesting 2.42 g HMB in free acid gel form [478]. It remained unchanged up to 2.5 hours after ingestion. In the same study, intake of 3 g leucine did manage to stimulate Akt phosphorylation. 4E-BP1 and S6K1 phosphorylation demonstrated a significant increase only 0.5 hours after HMB intake, while it showed a significant increase 1.5 hours after intake of leucine. The fractional synthetic rate (FSR; a reliable measure of protein synthesis) of muscle protein (the myofibrillar proteins) was also measured in this study. FSR increased by $\sim$70 % after HMB intake, and $\sim$110 % after leucine intake. However, the difference wasn't statistically significant. It also turned out that HMB intake inhibited muscle protein breakdown in an insulin-independent manner. If HMB inhibited muscle protein breakdown more than leucine (not measured), it could help explain why protein synthesis increased to a lesser extent. After all, fewer free amino acids would be released from protein breakdown to stimulate protein synthesis. Nevertheless, the question is whether the increase in FSR would be additive to that of a protein-rich meal with sufficient leucine.

6.2.2 Inhibition of proteasomal breakdown

The proteasome is a large protein complex that has a cylindrically shaped chamber in the center that consists of proteases. Proteins tagged with ubiquitin end up in this chamber, where they are subsequently degraded. This tagging is regulated by the ubiquitin system which consists of three components, E1, E2 and E3. The first component consists of the ubiquitin-activating (E1) enzymes. These hydrolyze ATP to activate ubiquitin by 'carrying' it. Ubiquitin is then transferred to ubiquitin-conjugating (E2) enzymes. The E2 enzymes then interact with ubiquitin (E3) ligases, which elect the proteins to be tagged with ubiquitin. Thus, they determine which proteins are labeled for degradation by the proteasome. Two major E3 ligases involved in muscle atrophy are muscle atrophy F-box (MAFbx, also known as atrogin-1) and muscle ring finger 1 (MuRF1) [56, 55]. These two E3 ligases are therefore often used as biomarkers for protein breakdown in muscle.

In an experimental model of cancer-induced weight loss in rats, HMB was able to inhibit proteasome activity, derived from chymotrypsin-like enzyme activity [419]. This inhibited protein breakdown, and an increase in soleus muscle mass of the rats was also found. Furthermore, a decrease in $E2_{14k}$ expression (an E2 enzyme) was found.

Other research shows an inhibition of the age-induced rise in MAFbx mRNA expression in rats [481]. An anti-catabolic effect of HMB was also observed in *in vitro* research employing L6 myoblasts [26]. The cells were incubated with dexamethasone, a glucocorticoid that stimulates the expression of MAFbx and MuRF1 and promotes muscle breakdown. The addition of HMB inhibited the increased expression of both these E3 ligases.

Nevertheless, not all research points in the same direction. A recent study looked at

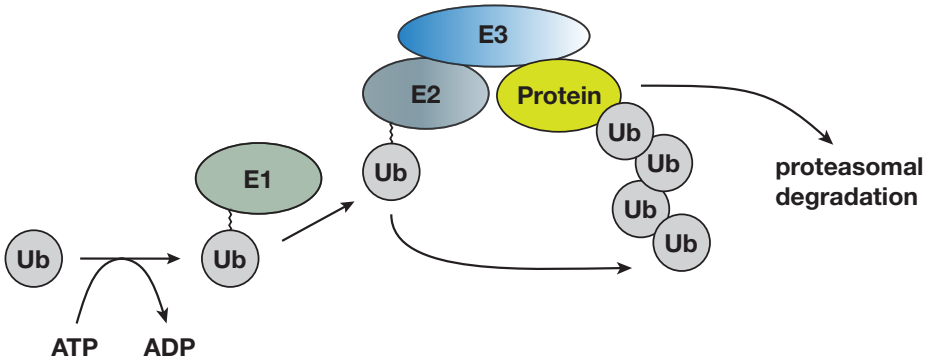


Figure 6.3: Proteasomal breakdown. E1 enzymes hydrolyze ATP to activate ubiquitin. These can then be carried over to E2 enzymes. After this, interaction can take place between E2 and E3 enzymes, such as MAFbx and MuRF1, to tag proteins with ubiquitin. The proteasome can then recognize these proteins and break them down. Figure based on Nguyen et al. [328].

the effects of leucine or HMB supplementation under two different conditions [32]. For example, the effect of both in combination with the administration of dexamethasone to rats was examined. Neither HMB supplementation nor leucine supplementation was able to counteract muscle atrophy of the soleus muscle. HMB did manage to inhibit the increase in MAFbx and MuRF1 expression; leucine, on the other hand, only inhibited MAFbx expression. The effect of both in combination with muscle immobilization was also examined. Leucine managed to minimize muscle atrophy, while HMB showed no effect. Leucine also minimized the expression of both E3 ligases, while HMB even led to an increase in MAFbx expression. Remarkably, a six-fold increase MAFbx expression was also found in the non-immobilized muscle, while leucine had no effect on this.

HMB supplementation did manage to inhibit muscle atrophy in another study in which dexamethason was administered to rats [165]. Importantly, however, this inhibition didn't become significant until eighteen days had passed; the aforementioned study only lasted seven days, so this effect may have been missed. In this study, researchers also looked at the effect of HMB on the expression of E3 ligases in L6 myoblasts incubated with dexamethason. The addition of HMB abolished the increase in MuRF1 and MAFbx mRNA expression [165].

As noted earlier, HMB can inhibit protein breakdown in humans in an insulin-independent manner [478]. This is interesting given that protein and carbohydrate intake inhibit protein breakdown by 50 % as a consequence of the resulting increase in insulin [24]. Without this rise in insulin, protein breakdown is virtually not inhibited. The question then is: would HMB have an additive effect to this? This has not been investigated to date. Additionally, the insulin-independent inhibition of protein breakdown might make it interesting to use in a diet involving periods of fasting, such as intermittent fasting. It could then be useful during these fasting periods in which insulin levels are low.

6.2.3 Improved membrane integrity

Cholesterol is an important constituent of membranes. It affects membrane integrity and fluidity. A sufficient and well-regulated concentration of cholesterol in the membranes is therefore essential for the well-being of cells. In addition, cholesterol is the precursor of steroids such as testosterone.

A cell can obtain its cholesterol in two ways. Either by endocytosis of LDL particles, or by *de novo* synthesis in the cytoplasm of the cell. The *de novo* cholesterol synthesis proceeds via the so-called mevalonate pathway. In this pathway, HMG-CoA is generated from acetyl-CoA. The generated HMG-CoA is then reduced to mevalonate by HMG-CoA reductase. Mevalonate subsequently goes through numerous chemical reactions to eventually form part of a cholesterol molecule. Multiple mevalonate molecules are required for the synthesis of a single cholesterol molecule.

The primary metabolic pathway of HMB appears to be its conversion to HMG-CoA, the precursor to mevalonate [332]. As a result of this, it's plausible that HMB supplementation can help safeguarding the cholesterol balance in a cell. However, no direct measurement of the effect of HMB supplementation on membrane integrity has been made to date. Some [331, 460, 484], but not all [482] studies show a decrease of creatine kinase in the circulation. Improved membrane integrity could potentially lead to less 'leakage' of creatine kinase into the circulation after resistance exercise. For the time being, this mechanism of action is based on little and indirect evidence.

6.2.4 Increased satellite cell activity

Stimulation of satellite cell activity might play a role in muscle hypertrophy and in any case in injury recovery, as discussed in Section 1.5. Research demonstrates that the addition of HMB to human and chicken myoblasts leads to an increase in satellite cell activity [262]. An increase in MyoD expression was also found, which is a biomarker used to get an idea of the number of active satellite cells. Additionally, an increase in the incorporation of labeled thymidine into the DNA was seen. These findings both indicate stimulation of myoblast proliferation. The addition of HMB also stimulated differentiation, as inferred from increased myogenin expression.

To determine the effect on satellite cell fusion, the researchers counted the number of nuclei of several hundred cells. The number of nuclei per cell increased with the addition of HMB. HMB thus stimulates proliferation, differentiation and fusion of myoblasts. The effect appears to be mediated by activation of the mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) pathway, and the PI3K/Akt pathway (see Section 4.2.1).

Another study also showed an increase in the proliferation and differentiation of satellite cells in rats during recovery from disuse atrophy after HMB administration [13].

6.2.5 Inhibition of autophagic-lysosomal degradation

Besides the proteasomal system, the autophagic-lysosomal system also regulates protein breakdown. With this system, a piece of cytoplasm is basically surrounded by a large number of vesicles that merge together to form an autophagosome. The autophagosome has an inner and outer membrane and directs its contents to the lysosome. The autophagosome then fuses with the lysosome, resulting in digestion of the autophagosome and its contents. The lysosomal lumen is a fairly acidic environment and contains a repertoire of hydrolases that promote degradation.

Autophagy is an important mechanism for cells to break down aged or damaged molecules and organelles, and to provide the cells with nutrients in the right place in times of need. Catabolic conditions stimulate this system, including administration of the glucocorticoid dexamethasone.

To date there appears to be only a single study which examined the effect of HMB on this [165]. HMB managed to inhibit lysosomal proteolysis, inferred from LC3-II, p62 and Bnip3 expression (biomarkers of autophagic-lysosomal activity) in L6 myoblasts exposed to dexamethasone. HMB likely inhibits this by reducing the activity of the FoxO3a protein (a transcription factor) which induces, among other things, the expression of autophagy-related genes [399]. This inhibition of FoxO activity is probably mediated by Akt. Akt phosphorylates and thereby deactivates FoxO proteins [502].

6.3 Clinical results

A lot of studies have been carried out to date to examine the effects of HMB supplementation on muscle strength and body composition. Problematic, however, is that the results of these studies don't really seem to align with each other. Moreover, they're often quite different in their design.

The trial by Nissen et al., as already briefly discussed in the introduction, demonstrated an increase in fat free mass (FFM) from day 14 to day 39 in the HMB group compared to the placebo group [331]. At the end of the trial, this difference was no longer significant. In addition, the HMB group also received a protein supplement while the placebo group didn't. Finally, the subjects were untrained.

A later trial by Kreider et al. examined the effect of a placebo, 3, or 6 g HMB daily in a trained population [266]. The subjects in this study had to have at least 1 year of experience with resistance exercise. Body composition was determined using dual-energy X-ray absorptiometry (DXA) at two points in time: before and 28 days after the supplementation and training period. No differences were found between the placebo and HMB groups. Strength was also determined by means of an 1-RM on the leg press and bench press. Again, no differences between groups were found after 28 days.

One reason why the results of Kreider et al. differed from those of Nissen et al. might be the training status of the subjects. A study by Nissen's group attempted to answer exactly this question by including both trained and untrained subjects in it [342]. The subjects in this trial followed a resistance exercise program three times a week for a total of four weeks. Subjects were divided into a placebo group or a HMB group (receiving 3 g daily). An increase in the 1-RM bench press was found in the HMB group compared to the placebo group, and a trend for an increase in FFM and a decrease in fat percentage (both $P = 0.08$, determined with a skin caliper). Remarkably, training status (untrained or trained) didn't appear to affect results.

Later research, in which Nissen was also involved, suggests that the effect of HMB supplementation on strength and body composition is enhanced when it's combined with creatine [245]. Or in other words: the effects are additive. The enrolled subjects weren't allowed to have followed a resistance exercise program in the six months before, and can therefore be considered untrained. They were divided into a creatine group (CR, 20 g daily for 7 days followed by 10 g daily), HMB group (HMB, 3 g daily), creatine + HMB group (CR/HMB), and a placebo group (PL). The study lasted (only) three weeks. Bioelectrical impedance analysis (BIA) was used to assess body composition. The effect

on body composition is shown in Table 6.2. The increase in LBM was significantly higher in CR compared to PL, and almost significant ($P = 0.08$) for HMB compared to PL. Because no significant CR $\times$ HMB interaction was found, and the increase in CR/HMB was even greater than that of the other groups, an additive effect might be presumed. Also noteworthy was that there was a large increase in fat mass in the CR/HMB group. The authors provide no explanation for this. Strength was determined by assessing the 1-RM of exercises that were part of the resistance exercise program (such as bench press and squat). The cumulative 1-RM strength gain was 37.5, 39.1 and 51.9 kg higher for CR, HMB and CR/HMB, respectively, compared to PL ($P = 0.001$). Again, no significant CR $\times$ HMB interaction was found, suggesting that the effect on strength of HMB and creatine supplementation is additive.

However, another study in which HMB (3 g daily) was combined with creatine (3 g daily) for six weeks found no effect on strength, power and anthropometrics (thickness of skin folds) in elite rugby players [334].

Outcome measure	PL	CR	HMB	CR/HMB
Weight (kg)	+1.00	+2.01	+1.34	+3.40
Fat mass (kg)	+0.17	+0.19	+0.08	+1.00
LBM (kg)	+0.85	+1.77	+1.24	+2.39

Table 6.2: Effect on bobody composition of placebo (PL), creatine (CR), HMB, and combined creatine and HMB (CR/HMB) intake. Fat mass and LBM were determined employing BIA. Results from Jowko et al. [245].

Other research also showed a significant increase in VVM in untrained men after 8 weeks of HMB supplementation (38 mg/kg bw; roughly 3 g daily) in combination with resistance exercise compared to placebo [156]. Remarkably, these increases were not found in the men who received a dose twice as high (76mg/kg bw). It is unclear why this higher dosage was less effective.

In the same trial, the 1-RM of ten exercises was also determined. No significant group-$\times$-time interaction was found for any of these ten exercises. However, some significant improvements were found in isometric and isokinetic strength measurements of the knee extensors at various angular speeds in the HMB groups.

A study with recreationally trained men showed no significant effect on body composition (assessed with a skin caliper) after nine weeks of HMB use [442]. HMB also had no effect on upper body strength, but did affect that of the lower body (1-RM leg extension). In a cross-over designed trial, HMB had no effect on strength of both the upper and lower body in trained rugby players [370].

Yet another study in elite rugby players also found no effect on strength of the upper and lower body after eleven weeks of HMB supplementation [306]. There was also no effect on fat percentage. Body weight did increase in the HMB group (+0.57 kg), while it decreased in the placebo group (-1.39 kg). Remarkably, this study found a negative effect on intermittent sprint performance in the HMB group versus the placebo group. However, the study suffered from a high dropout rate, which made the number of participants per outcome measurement quite limited. The high dropout rate is quite common among rugby players due to the intensity of their training schedules.

Finally, a controversial study by Wilson et al. has attracted a lot of attention [483]. The subjects were well accustomed to resistance exercise. For example, their squat, bench press and deadlift were on average 1.7, 1.3 and 2.0 times their body weight, respectively. The study lasted twelve weeks, following an undulating periodized resistance-training program. The 1-RM squat, bench press, and deadlift were used as outcomes for strength. Body composition was assessed using DXA. Additionally, muscle thickness of the quadriceps was determined using ultrasonography. So, what made this study so controversial? The results. The HMB group managed to gain 7.4 kg LBM while losing 5.4 kg of fat. 1-RM squat increased from 143.7 to 179.9 kg (+25 %), 1-RM bench press from 112.4 to 125.2 kg (+12 %), and 1-RM deadlift from 170.3 to 198.4 kg (+16 %). Quadriceps muscle thickness increased by 14 %. It's very hard to imagine that there is any truth to such incredible results.

Another study also conducted by Wilson's group, in which HMB was combined with ATP supplementation, showed similar astonishing results [286]. It should be noted that both studies were sponsored by Metabolic Technologies Inc, a supplement company that sells HMB and was founded by Steven Nissen. Several of the co-authors were employed by this company. The inconsistency of the results of this study compared to other data from the literature has thus led to quite some criticism [232].

6.4 Safety

An article by Steven Nissen et al. that included 9 trials reviewed the safety of the supplement [330]. In total, over 100 subjects received HMB at a dosage of 3 g daily for 3 to 8 weeks. No negative effect was found on serum lipids, markers of liver function and damage, markers of renal function, serum electrolyes, haematological values and blood pressure. Quite in fact, a decrease in total cholesterol (-5.8 %), LDL-cholesterol (-7.3 %) and systolic blood pressure (-4.4 mmHg) was observed. It's unknown whether this decrease in cardiovascular risk markers also decreases actual cardiovascular disease. The subjects had also undertaken questionnaires to assess side effects, and these also came back clear. A later study in female elite judokas on an energy-restricted diet, showed an increase in total cholesterol (+12 %) after 3 days of supplementation [230]. The short supplementation duration might have played a role in this, as this effect might have been transient.

A 2013 systematic review also states that HMB supplementation is safe [315]. The authors do report that in two of the reviewed trials [371, 98] an increase in blood urea nitrogen (BUN) was found. In both studies, however, HMB was combined with the amino acids arginine and glutamine, which may be responsible for the detected increase. Other studies don't report an increase in BUN.

To date, long-term studies of HMB are lacking. Therefore, some caution is advised with chronic use of the supplement.

6.5 Conclusion

HMB is an important metabolite of leucine and appears to be tightly involved in the regulation of protein synthesis and degradation. The amount of HMB that is produced endogenously is quite limited and is estimated to be less than a gram daily, and is dependent on the amount of protein in the diet. For that reason, supplementation could possibly have

a positive effect on body composition and muscle strength. Its actions might be attributed to stimulation of mTOR signaling, inhibition of protein degradation by the proteasome, improvement of membrane integrity, stimulation of proliferation, differentiation and fusion of satellite cells, and modulation of the autophagic-lysosomal system.

A substantial number of clinical studies have investigated the effects of HMB supplementation in both athletes and untrained subjects. The effective dose used for this is commonly 3 g daily. The results of the clinical studies vary widely: from either no effect at all, to an enormous increase in strength and LBM. It's unclear why the results between studies are so inconsistent. Nevertheless, many of the positive studies have been sponsored by the supplement company that's behind HMB. The studies with extremely positive results have received well-founded critique in the literature.

The supplement scores well in terms of safety. To date there is no indication that HMB is detrimental to health in the short term. However, long-term studies are lacking.

7. Caffeine

7.1 Introduction

Caffeine (1,3,7-trimethylxanthine) is common in most people's daily diet. This is largely due to the presence of the substance in coffee. In addition, caffeine is found in tea, chocolate milk, soft drinks such as cola, and energy drinks. The use of caffeine in the form of coffee and tea has a rich history [154]. Both have been consumed for hundreds of years and the use of coffee or tea is a part of many cultures. Nowadays, a large part of the caffeine intake among the Dutch population can also be attributed to the increasing consumption of energy drinks, which were introduced in the 1980s and are particularly popular among young people. Nevertheless, caffeine consumption is still higher in those 31 to 69 years of age (500–549 mg daily for men and 462–505 mg daily for women) than in those aged 19 to 30 years (316 mg daily for men and 281 mg daily for women), according to the consumption of caffeinated foods in the 2007-2010 Dutch Food Consumption Survey [459]. Table 7.1 lists the caffeine contents of some dietary sources.

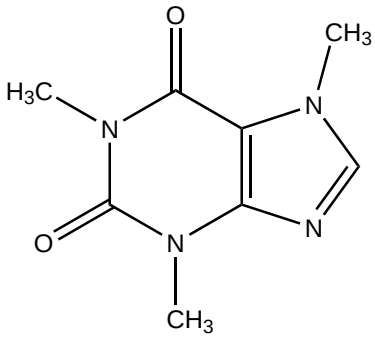


Figure 7.1: Structural formula of caffeine.

Caffeine is also popular among athletes as a performance-enhancing agent. Doses of ∼3–6 mg/kg bw are used for this purpose [170], an intake comparable to what most adults consume daily. In the context of sports performance, caffeine is also one of the best

Product	Unit	Caffeine (mg)
Coffee	Cup (150 mL)	40–180
Tea	Cup (150 mL)	25–50
Soft drinks	Can (250 mL)	20–40
Energy drinks	Can (250 mL)	30–85
Chocolate	Bar (100 g)	5–20

Table 7.1: Caffeine contents of some dietary sources. Data taken from [294].

researched supplements.

7.1.1 Absorption

Caffeine, as a supplement, is taken orally in a capsule or as a powder. In the diet it's usually part of coffee, tea, or soft drinks. It's primarily absorbed by the small intestine, but about 20 % is already absorbed by the stomach [96]. Absorption takes place quite quickly. In a small-scale ($n = 10$) pharmacokinetical study, 5 mg/kg bw caffeine was given orally to subjects [51]. The mean time to reach the peak caffeine plasma concentration after ingestion was 29.8 minutes (with a standard error of 8.1). In the same study, participants also received the same dose intravenously, in order to draw a comparison with oral ingestion to determine its bioavailability. This showed that caffeine is fully bioavailable orally and thus there's no first-pass effect of the substance. The peak plasma concentration that was reached ranged from 6.9 to 16.1 μg/mL with a mean value of 9.9 μg/mL. The half-life averaged at 4.5 hours, with a range of 2.7–9.9 hours. This indicates there's a strong inter-individual variability in its half-life.

Other research has also looked at the pharmacokinetics of caffeine when taken as coffee, cola or chocolate [321, 281]. Mumford et al. gave 7 subjects a dose of 72 mg caffeine (averaging 1.01 mg/kg bw) as a capsule, cola, or chocolate [321]. (The chocolate also contained the dimethylxanthine theobromine.) This study also found a peak in the caffeine plasma concentration after half an hour in those receiving a caffeine capsule. When administered cola or chocolate, this peak wasn't reached until after an average of 120 minutes. This might be because sugar slows down the gastric emptying of caffeine [96] and a solid substance, such as chocolate, also takes longer to reach the small intestine. However, another study found no delayed absorption of caffeine after consumption of cola [281]. Nevertheless, the cola in this study contained no sugar (and the caffeine dose was higher at 400 mg). The same study also found that caffeine from coffee wasn't absorbed with a delay.

Caffeine is sufficiently non-polar to diffuse across cell membranes, and only a small part of it gets bound by a protein in the circulation. About one third is bound to albumin, both in the young and the elderly (who generally have a lower serum albumin concentration) [50]. This allows caffeine to easily spread over all the water the body contains. The steady-state volume of distribution is between 0.5 and 0.8 L/kg [294]. While it's believed that caffeine primarily moves across cell membranes by passive diffusion, research has demonstrated that some carrier-mediated transport (see Section 3.2.2) across the blood-brain barrier also takes place [304].

In another study it was found that the course of the plasma concentration over time was

comparable to the concentration in the extracellular fluid of the subcutaneous abdominal adipose tissue [425]. However, the plateau concentration between the two showed no correlation. As such, the concentration to which cells are exposed can deviate from the concentration as measured in the blood plasma.

7.1.2 Metabolism and excretion

Caffeine is extensively metabolized by the liver. These reactions are primarily catalyzed by members of the cytochrome P450 enzyme system, of which CYP1A2 in particular. The first metabolic step is a demethylation reaction. This demethylation can take place on the first, third or seventh nitrogen atom. This yields the metabolites: 3,7-dimethylxanthine (theobromine), 1,7-dimethylxanthine (paraxanthine), and 1,3-dimethylxanthine (theophylline), respectively. The fractional conversion from caffeine to paraxanthine averages 76.6 %, to theobromine 10.8 % and to theophylline 3.7 % [277]. These reactions are catalyzed by the enzymes CYP1A2 and CYP2E1 [294]. CYP1A2 is quantitatively by far the most important in this regard. After the first demethylation another demethylation reaction takes place. This transforms the dimethylxanthines into monomethylxanthines. This reaction is also mainly catalyzed by CYP1A2, although there are some other CYP enzymes which do this too. Finally, an oxidation step is carried out to form uric acids. The dimethylxanthines can also be oxidized directly, however. Additionally, there are some other metabolic paths that caffeine can follow in which N-acetyl transferases, xanthine oxidase and other CYP enzymes are involved. Nevertheless, these play a very small role in quantitative terms and will not be discussed further here.

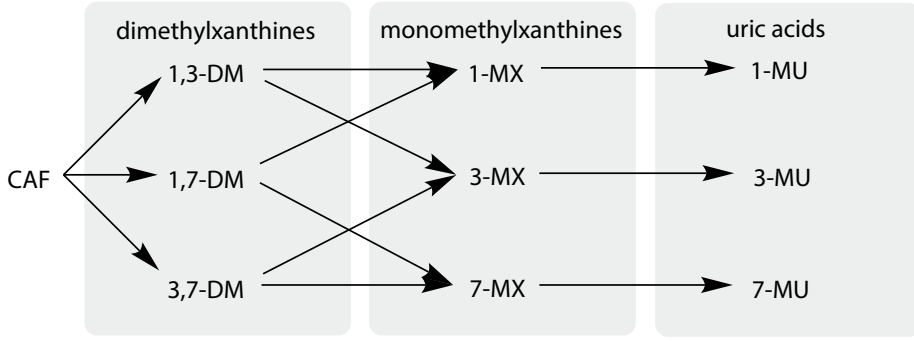


Figure 7.2: Simplified representation of caffeine metabolism. Caffeine is demethylated to one of three possible dimethylxanthines, after which these can be further demethylated to form one of the three possible monomethylxanthines. The monomethylxanthines are then oxidized to form uric acids. The dimethylxanthines can also oxidized to form uric acids (not shown). Abbreviations: CAF, caffeine; 1,3-DM, 1,3-dimethylxanthine; 1,7-DM, 1,7-dimethylxanthine; 3,7-DM, 3,7-dimethylxanthine; 1-MX, 1-methylxanthine; 3-MX, 3-methylxanthine; 7-MX, 7-methylxanthine; 1-MU, 1-methyluric acid; 3-MU, 3-methyluric acid; 7-MU, 7-methyluric acid.

The half-life varies from approximately 2.5 hours to 10 hours at doses less than 10 mg/kg bw [51, 294]. Higher doses can lead to a longer half-life as a result of saturation

of the metabolic pathways. A polymorphism of the *CYP1A2* gene is known that affects the rate of metabolism of caffeine. This polymorphism is a C-to-A transversion located in the first intron of the *CYP1A2* gene. People who carry this polymorphism in both alleles of the gene (homozygote) metabolize caffeine faster than people who don't carry this polymorphism or only carry it on a single allele (heterozygote) [392]. As a result, homozygotes will have lower concentrations of caffeine in the circulation after caffeine ingestion than heterozygotes and non-carriers.

Various external factors also affect the activity of the CYP1A2 enzyme which might further help explain the variability in the observed half-life of caffeine. This includes smoking, gender, BMI, pregnancy and phase of the menstrual cycle in women, as well as dietary factors such as the consumption of alcohol, grilled meat and grapefruit juice [86]. Exercise doesn't appear to affect CYP1A2 activity. The clearance of caffeine is slower towards the end of the luteal phase in women [271] and estrogen-containing contraceptives also delay clearance [4]. This might lead to the accumulation of caffeine with repeated dosing and prolongs its efficacy. Estrogen replacement therapy in postmenopausal women also slows down caffeine metabolism [358]. This suggests that estrogen is directly responsible for this effect: it's likely that estrogen inhibits CYP1A2 activity.

The metabolites of caffeine are cleared by the kidneys in the urine. Only a small fraction (< 3 %) of ingested caffeine leaves the body unchanged in the urine [47]. The caffeine concentration in urine is approximately equal to that of the free fraction present in the blood. This implies that caffeine is reabsorbed in the renal tubules until equilibrium with the free plasma fraction is reached.

7.2 Mechanism of action

The most important mechanism of action of caffeine stems from its capacity to bind to adenosine receptors without activating them [170]. As such, it acts as a competitive receptor antagonist and thus prevents binding of the physiological ligand: adenosine.

Adenosine is a common adenine nucleoside in the body. The molecule binds to adenosine receptors located on the plasma membrane of cells. Four different isoforms of these receptors are known. Because adenosine needs to bind to its receptor on the cell surface, the effects of adosine depend on its extracellular concentration. Adenosine can be generated both intracellularly and extracellularly. Extracellularly, it's produced from adenosine triphosphate (ATP). First, ATP needs to be converted to adenosine monophosphate (AMP). Subsequently, AMP can be hydrolyzed to form adenosine. Intracellular, in addition to also being produced from ATP, it's also formed from S-adenosylhomocysteine. The adenosine concentration rises sharply when ATP degradation exceeds ATP synthesis. After all, under such conditions more AMP will be formed which can function as a substrate to be hydrolyzed to adenosine. This takes place, for example, in muscle tissue during vigorous exercise or under extreme conditions such as ischemia or hypoxia of a tissue, which impedes aerobic energy metabolism. Adenosine receptor activation is involved in many biological functions, including sleep regulation, alertness and lipolysis. Caffeine interferes with this normal regulation by, as it were, pushing adenosine out of the picture by occupying its receptors without activating them. This leads to, among other things, the well-known effect of increased alertness, but also the ergogenic effects. Adenosine receptors are distributed throughout the body and are present in for example the brain, fat cells, and muscle cells. Because multiple adenosine receptor isoforms exist, which have

varying, sometimes opposing, functions, it has proven difficult to unravel the mechanisms underlying the ergogenic effect of caffeine. Nevertheless, some mechanisms have been proposed, such as a shift in substrate consumption towards fatty acids, stimulation of sodium potassium pump activity, and a decreased perception of exercise and pain.

7.2.1 Alteration of substrate utilisation

The first study demonstrating an improvement in sports performance after caffeine ingestion looked at the effect of it on time to exhaustion on a bicycle ergometer [104]. It showed caffeine to be effective in increasing the time to exhaustion. An increase in the plasma concentration of free fatty acids was also seen after caffeine intake. Indeed, caffeine leads to an increase in noradrenaline and adrenaline release [385] and it's also plausible that antagonism of the A1 isoform of the adenosine receptor on adipocytes stimulates lipolysis [207, 435].

Moreover, the intake of caffeine caused a decrease in the respiratory exchange rate (see Box 7.1). This decrease suggested that there was an increase in fat oxidation. Indeed, an increased plasma concentration of fatty acids causes many tissues, including skeletal muscle, to burn more fat. This phenomenon, in which substrate utilisation shifts from glucose to fatty acids, is also called the Randle cycle. Consequently, it can be speculated that the increase in fatty acid oxidation spares glycogen, which, in turn, could lead to the ergogenic effect under certain conditions. However, several follow-up studies failed to demonstrate a glycogen-sparing effect of caffeine [176]. Today, this is therefore no longer considered an underlying mechanism of action of caffeine's ergogenic effect (with perhaps some very specific exceptions).

Box 7.1

The respiratory exchange ratio is the ratio between the amount of carbon dioxide that has been produced and the amount of oxygen that has been reduced (and thus consumed). This ratio is determined by measuring the gas exchange at the mouth. This value is a good approximation of the respiratory quotient. The respiratory quotient yields the same ratio, but at the cellular level.

The ratio thus provides information about the substrate consumption for the production of energy. A ratio of 1 means that the amount of carbon dioxide produced is equal to the amount of oxygen consumed. A ratio lower than 1 means that more oxygen molecules are consumed than that carbon dioxide molecules are produced. Completely burning a glucose molecule costs 6 oxygen molecules and produces 6 carbon dioxide molecules. The ratio of someone burning glucose only would therefore be 1. However, the carbons of fat (and proteins) are less oxidized than those of glucose. As a result, more carbon dioxide molecules are produced per oxygen molecule when burning these for energy. For example, complete oxidation of the fatty acid palmitate gives a ratio of 0.7: 23 oxygen molecules are needed and only 16 carbon dioxide molecules are produced.

When an intervention results in a lower respiratory exchange rate, the intervention has probably led to an increase in fatty acid oxidation. At rest, in a fasted state, the respiratory exchange rate is about 0.8.

7.2.2 Stimulation of sodium potassium pump activity

The contraction of a muscle is initiated by an action potential (see Section 1.3). Rapid depolarization of the sarcolemma results from opening of sodium channels. During the

subsequent repolarization phase, potassium channels open which leads to an efflux of potassium. With repeated depolarizations, the cell loses a tiny bit of potassium each time. This results in an increase in the extracellular potassium concentration. This negatively impacts the excitation-contraction coupling (and therefore muscle contraction) and could also be a cause of muscle fatigue [380, 10, 99]. The sodium potassium pumps can restore the balance. Caffeine, and other methylxanthines, are capable of stimulating sodium potassium pump activity [282]. Thus, this could improve excitation-contraction coupling and aid in counteracting musscle fatigue.

Box 7.2

In 1996, Vanderberghe and his colleagues at the Catholic University of Leuven investigated whether caffeine supplementation in combination with creatine led to higher intramuscular creatine levels than creatine alone [461]. Creatine is transported across the sarcolemma by the creatine transporter (CrT). The CrT is a Na^+- and Cl^--dependent antiporter (see Section 8.1.2). A larger Na^+ gradient might thus increase the absorption of creatine by muscle cells. Since sodium potassium pumps increase this gradient, and caffeine stimulates the activity of these pumps, Vandenberghe and colleagues hypothesized that the addition of caffeine to a creatine loading phase would lead to even greater intracellular creatine levels. However, the clinical study showed that the addition of caffeine had no effect on the intracellular creatine concentration resulting from creatine supplementation.

7.2.3 Decreased perception of exertion and pain

Experiencing pain can lead to decreased sports performance. Pain signals the brain that tissue damage has occurred or might occur (or at least that's largely how it's interpreted). Pain arises from the stimulation of so-called nociceptor that thank their name to the Latin word *noxa* (= damage). Nociceptors are the free nerve endings of nocisensoric neurons, which pass the signal back to the spinal cord. In the spinal cord, it switches to ascending secondary neurons, which further pass it on to the brain. Nociceptors can sense different stimuli and propagate this along the nocisensoric neurons. For example, they ensure super-fast propagation when you accidentally put your hand in a stream of red-hot water, which causes a withdrawal reflex.

Important in the context of sports performance are, among other things, the chemical stimuli that arise during intense exercise. This can create a (characteristic) painful experience in the legs during running or cycling. Adenosine receptor activation might play an important role in the sensation of pain by a chemical stimulus [404]. As mentioned earlier, the extracellular concentration of adenosine increase with intensive exercise. This could be a cause of pain with such exercise. As an adenosine receptor antagonist, caffeine might be able to counteract this and thus improve sports performance.

The first study investigating the effect of caffeine on muscle pain during exercise was published in 2003 [318]. Caffeine ingestion (10 mg/kg bw) indeed led to lower pain scores when cycling at moderate intensity (60 % VO_2 peak). Follow-up research from the same lab suggests that this reduced sensation of pain by caffeine follows a linear dose-response function in men [335]. This dose-response function was not found in women [317]. It is unclear what could cause this sex difference. There might also be a pain level above which caffeine no longer has an effect [172]. Or in other words: for a given amount of pain, caffeine can improve performance as a result of its pain-reducing effect. However,

when the exercise leads to too much pain, caffeine's pain-reducing effect is nullified. Nevertheless, several studies show an improvement in sports performance at high intensity. This suggests that the ergogenic effect, at least at high intensities, doesn't result from caffeine's pain-reducing properties.

Closely related to the perception of pain is the perception of exertion. Perceived exertion involves the collective integration of afferent feedback from cardiorespiratory, metabolic, and thermal stimuli and feed-forward mechanisms to enable an individual to evaluate how hard or easy an exercise task feels at any point in time [144]. A 2005 meta-analysis showed that caffeine decreased the rate of perceived exertion (RPE; a scale for perceived exertion [62]) by 5.6 % during various sports such as cycling, running, swimming and rowing [130].

However, the effect of caffeine supplementation on RPE is less clear when examining research that focuses on resistance exercise. In one study, subjects were required to perform a variety of resistance exercises at 60 % of their 1-repetition maximum (1-RM), including bench press, deadlifts, and squats [135]. Caffeine supplementation resulted in an increase in the number of repetitions, as well as a lower RPE rating relative to a placebo. However, other comparable trials didn't demonstrate the latter [180, 227, 23, 113, 109].

It should be noted, however, that most research does show a performance-enhancing effect (e.g. more repetitions). It's therefore perhaps not that surprising that the RPE remains unchanged: the subjects are performing at a higher level that would otherwise likely push RPE higher too.

7.3 Clinical results

Many studies have evaluated the ergogenic effect of caffeine on performance in endurance and high-intensity sports (1–60 min, such as swimming, rowing, and middle-/long-distance running) [75]. Caffeine supplementation is effective in these sports. It's less clear what the effect of caffeine is for brief strength events. Most evidence indicates that the effect of caffeine supplementation on muscle strength (1-RM) and muscle endurance (repetitions to failure [RTF]) is small. Some studies find no statistically significant effect [22, 479, 138, 447], while others do—but the effect is often quite small [227, 169, 134].

Astorino et al. investigated the effect of a 6 mg/kg bw dose of caffeine on 1-RM bench press and leg press, and RTF at an intensity of 60 % 1-RM [22]. A total of 22 subjects with resistance exercise experience participated in this double-blind, placebo-controlled crossover trial. No statistically significant effect was found on either the 1-RM or RTF measurements, although there appeared to be an increase in the number of repetitions in the RTF measurements and resultingly a higher absolute load (11–12 % increase). It's possible that this didn't reach the threshold for statistical significance due to the small sample size.

A comparable study was conducted by Williams et al. [479]. This study was also double-blind, placebo-controlled and crossover designed, with a total of 9 trained subjects. The caffeine dose in this study was lower (300 mg, approximately 3.6 mg/kg bw). The researchers looked at the effect on strength (1-RM) and endurance (RTF at 80 % 1-RM) of the upper body (bench press and lat pulldown). This study too didn't find a statistically significant effect, although an increase in the absolute load was found here as well. The absolute load of the bench press increased by 10 % and that of the lat pulldown by 5 %. Again, the small sample size might be the reason for the lack of a statistically significant

effect. Nevertheless, a recent study by Trexler et al. also found no significant effect of caffeine supplementation (300 mg) on muscle strength (1-RM) and endurance (RTF at 80 % 1-RM) in a trained population [447]. Eckerson et al. demonstrated similar results in trained subjects that were given 160 mg caffeine [138]. Nevertheless, any ergogenic effect of caffeine might have been minimized—or nullified—as a result of the low dose, given that it's assumed one would need at least a dose of 3 mg/kg bw for an optimal effect.

In a crossover trial by Hudson et al., subjects performed four sets of leg extensions and arm curls to failure [227]. The used weight was based on an estimated 12-RM. The caffeine group in this study received a dose of 6 mg/kg bw. A significant increase in the total number of repetitions compared to the placebo group was noted. However, this significant increase was only found in the first set of a series of four. Another crossover trial by Duncan et al. also found an increase in muscle endurance after caffeine ingestion [134]. The subjects in this study had 9.5 years of resistance training experience on average. Muscle endurance was determined by RTF at 60 % 1-RM on the bench press, deadlift, prone row, and squat. After taking caffeine, 1.5 more repetitions were made on average. Remarkably, the caffeine dose that was used in this study was relatively low, only 179 mg.

Goldstein et al. investigated the effect of caffeine on upper body strength in fifteen women with resistance training experience [169]. Caffeine ingestion (6 mg/kg bw) led to a significant increase in the 1-RM on the bench press. However, the increase was very small (+0.8 kg). RTF at 60 % 1-RM was also determined, but this remained unchanged after caffeine ingestion.

The results in the literature appear variable, but perhaps this is due to the small sample sizes in these studies. Caffeine supplementation appears to have an acute effect on muscle endurance, allowing for more repetitions to be made at a given intensity and resulting in a higher absolute load. Caffeine might also increase strength, as assessed by a 1-RM, to a small extent.

7.4 Safety

Since a huge part of the world population consumes caffeine on a daily basis, there's a wealth of scientific research available about its safety. Caffeine is a substance with an acute effect. People often feel more alert within an hour after ingestion. This rapid effect is also reflected in, for example, blood pressure. A single dose of 200–250 mg in normotensive (having a normal blood pressure) people is reported to increase systolic blood pressure by 3–14 mmHg and diastolic blood pressure by 4–13 mmHg [333]. This increase can be measured within 0.5 hours after intake, peaks after approximately 1–2 hours, and can last for up to 2–4 hours. An increase also occurs in hypertensive (having a high blood pressure) people. For example, an increase in systolic blood pressure of 8.1 mmHg and an increase in diastolic blood pressure of 5.7 mmHg is found in hypertensive patients after taking 200–300 mg caffeine [311]. A small study also shows a slight increase in systolic blood pressure in normotensive men before, during and after resistance exercise after ingestion of caffeine (6 mg/kg bw) compared to a placebo [21].

Caffeine also causes myocardial (heart muscle) blood flow to decrease during exercise [209]. This sounds more alarming than it is. The European Food Safety Authority (EFSA) concludes that there is no clinically relevant decrease in coronary blood supply (blood supply to the heart) with doses up to 200 mg in otherwise healthy adults [441]. There's insufficient data about higher doses and the EFSA also states that the effect on blood

pressure with doses up to 200 mg is of low clinical relevance. Notably, chronic coffee consumption (>3 cups daily) shows no association with an increased risk of hypertension [504]. Chronic caffeine consumption therefore does not appear to increase the risk of cardiovascular disease [485].

Caffeine also has a diuretic effect, or in other words, it increases urine output. This diuretic effect is the result of its natriuretic (enhanced sodium excretion) effect on the kidneys. A meta-analysis shows that this effect at rest, however, is quite small in men, although the effect is more prominent in women [503]. Remarkably, the diuretic effect of caffeine is virtually non-existent when combined with exercise. The anti-diuretic effect of exercise probably negates the diuretic effect of caffeine. In summary, there's no concern for dehydration, nor need to increase fluid intake, when taking caffeine in conjunction with exercise.

Box 7.3

When you exercise and start to sweat, the blood plasma volume will decrease and consequently the blood osmolality will increase. In the brain, more specifically the hypothalamus, the increase in blood osmolality is picked up by osmoreceptors. In order to maintain a correct blood osmolality, your body will put some things into action. The hypothalamus transmits a signal to the posterior pituitary, which, in turn, releases a substance called antidiuretic hormone (ADH, also known as vasopressin) into the circulation. ADH then acts on receptors in the kidneys to enhance water reabsorption (anti-diuretic effect). This way the body limits fluid loss. Exercise also activates the renin-angiotensin-aldosterone system (RAAS) after a few minutes. Activation of RAAS causes, among other things, an increase in blood aldosterone levels. Aldosterone also acts on receptors in the kidneys, causing them to reabsorb more sodium (antinatriuretic effect) and therefore more water.

The 'alerting' effect of caffeine has the downside that it can make you sleep worse. It can extend sleep latency (the time required to fall asleep), shorten the total sleep time, and decrease the perceived quality of sleep [97]. For that reason it's advisable to refrain from taking caffeine just before going to sleep. As discussed in Section 7.1.2, caffeine's half-life varies widely, from about 2.5 hours to 10 hours. In those people whose caffeine metabolism is slow, caffeine taken during the afternoon might even still have a negative effect on sleep at night. The negative effect on sleep is more prominent in those who have a low habitual intake of caffeine compared to those who have a high habitual intake [211]. It's advisable to lower the dose, or ingest caffeine at an earlier time, if sleep problems occur. That way the effect is largely worn off when it's time to sleep.

In summary, the EFSA concludes that a daily caffeine intake of up to 400 mg (approximately 5.7 mg/kg bw for a 70 kg adult) is safe for healthy individuals, excluding pregnant women [441]. Pregnant women should limit their intake to 200 mg daily. Single doses of up to 200 mg (approximately 3 mg/kg bw for a 70 kg adult) are considered safe for healthy individuals. Such a dose is also considered safe when ingested less than 2 hours before intense physical exercise. The EFSA doesn't comment on higher single doses. However, the studies using single doses up to 6 mg/kg bw report no serious side effects. Many do notice a 'rushed' feeling or an increased heart rate at higher doses.

7.5 Conclusion

Caffeine is widely consumed around the globe, and its intake is also high in the Netherlands. Caffeine is absorbed fairly quickly after ingestion and the half-life varies widely from one person to the next. Factors that play a role in this include smoking, gender, BMI, pregnancy and phase of the menstrual cycle in women, but also dietary factors such as the consumption of alcohol, grilled meat, and grapefruit juice. Via the blood circulation caffeine reaches various body tissues, where it can then bind to adenosine receptors. Because caffeine binds to these receptors without activating them, it effectively 'disables' the physiological ligand adenosine. Caffeine owes its effect to this mechanism. In particular, it lowers the perception of exertion and pain during exercise. This may be why at a given intensity, more repetitions can be performed. As such, it promotes muscle endurance. The effect on 1-RM strength increases is quite small. An effective dose for this purpose is 3–6 mg/kg bw. This boils down to 210–420 mg for an adult weighing 70 kg. An intake of up to 400 mg daily, or 5.7 mg/kg bw for a 70 kg adult, is considered to be completely safe by the EFSA. A single dose of 200 mg, or about 3 mg/kg bw for a 70 kg adult, is also considered safe by the EFSA.

8. Creatine

8.1 Introduction

Roger C. Harris and his colleagues showed in 1992 that repeated dosing of large amounts of creatine (5 g creatine monohydrate four or six times a day for more than 2 days) led to an increase in human quadriceps femoris creatine concentration [196]. Part of the creatine accumulated in the form of creatine phosphate. Given its important role in the energy metabolism of muscle cells, among other things, these results didn't go unnoticed by athletes for long. As such, it was in the early nineties that creatine supplementation made its rise and became a very popular dietary supplement. Today it's still extremely popular among athletes of different disciplines, but in particular athletes who wish to increase muscle mass or gain strength.

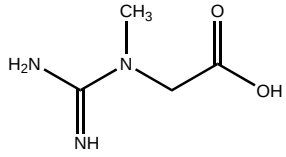


Figure 8.1: Structural formula of creatine. Under physiological conditions the carboxyl group (-COOH) is deprotonated ($pK_a = 3.8$) and the imino group (=NH) is protonated ($pK_a = 12.7$).

The popularity of creatine can be attributed to several aspects, for example an enormous amount of clinical research has demonstrated that creatine has an ergogenic effect in high-intensity training, such as resistance exercise. Numerous studies also show a positive effect on lean body mass. In addition, creatine is incredibly cheap and has an excellent safety profile.

Even without creatine supplementation, most people consume reasonable amounts of it as part of their diet. Creatine is mainly present in muscle tissue, and creatine can therefore be found in the meat that people eat.

8.1.1 Biosynthesis

Creatine spontaneously, and irreversibly, decomposes to creatinine. Creatinine is excreted by the kidneys in the urine. Every day, about 1.7 % of the total amount of creatine in the body decomposes to creatinine. For a 70 kg adult man between 18 and 29 years old, this amounts to roughly 1.7 g daily (23 mg/kg bw/day) [426]. This number is slightly lower for the elderly and women, because they have relatively less muscle mass. The amount of creatine in the body is largely a reflection of the amount of muscle mass. This is because the human body consists for a large part of muscle mass, and muscle has a high concentration of creatine. As a consequence, someone who carries a lot of muscle mass will therefore also have a higher creatine turnover—after all, there is more creatine present in the body to decompose to creatinine.

Given this continuous decomposition of creatine to creatinine and its subsequent urinary excretion, the body must compensate by replenishing its creatine stores somehow. This can be done in two ways. One being by obtaining creatine from the diet. In which it's mainly present in meat and fish. The other way would be by creatine biosynthesis (see Figure 8.2). Or in other words: the body synthesizing the creatine itself. The human body can synthesize creatine from the two amino acids glycine and arginine. These two amino acids form the building blocks for the direct precursor of creatine: guanidinoacetic acid. First, a guanidine moiety from arginine is transferred to glycine to form guanidinoacetic acid and ornithine. This reaction is catalyzed by arginine aminotransaminase (AGAT). Subsequently, a methyl group is transferred from S-adenosylmethionine (SAM) to guanidinoacetic acid, forming creatine and S-adenosylhomocysteine. This reaction is catalyzed by guanidinoacetate methyltransferase (GAMT). Methionine is converted, and thereby 'activated', to SAM with the energetic help of ATP, a reaction catalyzed by methionine-adenosyl transferase. SAM is involved as a methyl donor in numerous processes [280]. The first reaction of the biosynthesis of creatine, forming guanidinoacetic acid, is considered the flux-generating step [471]. AGAT thus functions as the flux-generating enzyme.

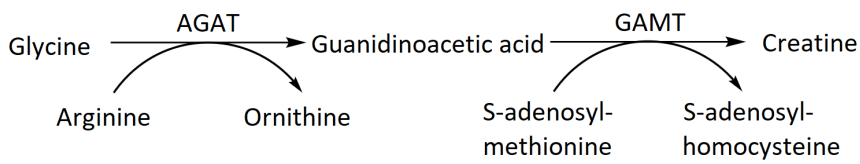


Figure 8.2: Creatine can be endogenously synthesized by a two-step mechanism. The enzyme AGAT catalyzes the first reaction, forming guanidinoacetic acid and ornithine from glycine and arginine. Guanidinoacetic acid then forms the substrate for the second reaction catalyzed by GAMT. S-adenosylmethionine functions as a methyl donor in this reaction which forms creatine.

About 20 % of guanidinoacetic acid synthesis takes place in the kidneys [139]. Subsequently, transport to the liver takes place, where the guanidinoacetic acid is converted into creatine as a result of the high AGAT activity there. The remaining guanidinoacetic acid is probably produced in the liver and pancreas [489].

Creatine supplementation leads to a decrease in AGAT activity and AGAT mRNA. This leads to inhibition of creatine biosynthesis [139, 250]. This is a form of feedback inhibition: as soon as enough end product (creatine) is present, the flux-generating step (transfer of the guanidine group to glycine) of the metabolic pathway is inhibited.

8.1.2 Absorption

Creatine is supplemented orally after which it enters the stomach via the esophagus. The stomach has a low pH value (it's very acidic). In the past, there have been concerns about the degradation of creatine to creatinine due to the low stomach pH. However, these concerns have turned out to be unfounded. The half-life of creatine, degrading to creatinine, is 55 days at a pH value of 1.4, 7.5 days at a pH value of 3.7, and 40.5 days at a pH value of 6.8 [350]. Since creatine will only be present in the stomach for a fraction of the time of these half-lives, no significant degradation to creatinine will occur.

Creatine enters the small intestine from the stomach. It then relies on active transport for its absorption. Transporters that are able to transport creatine across the intestinal wall have been found in rodent ileum and jejenum [443, 348]. In addition, creatine transporters are located on both the apical [443] (facing the intestinal lumen) and basolateral [339] membrane of enterocytes.

The transporter that facilitates transport of creatine across the cell membrane is called the creatine transporter (CrT). The CrT is a Na^+- and Cl^--dependent antiporter that couples the concentration gradient of these ions (high extracellularly, low intracellularly) to the inward transport of creatine against its concentration gradient. This is a form of secondary transport (described in Section 3.2.2). The K_d value of the human CrT is estimated to be between 15 and 77 μM. Creatine plasma concentrations are usually around 50–100 μM [420]. Assuming the low end of the K_d range and the high end of the plasma concentration range, would lead to the conclusion that creatine transport is clearly saturated. However, as we know, creatine supplementation shows a marked increase in the intracellular creatine concentration of muscle tissue, as demonstrated in 1992 by Roger C. Harris and his colleagues [196]. An observation that has also been made in countless subsequent studies. As such, it seems likely that creatine transport is normally not saturated, or its transport activity quickly changes with higher plasma concentrations.

Insulin enhances the accumulation of creatine in muscle tissue, probably by stimulating creatine transport [179, 178, 427, 357]. This is likely because of its stimulating effect on sodium potassium pump activity, probably because insulin leads to translocation of sodium potassium pumps to the sarcolemma [432]. This, in turn, stimulates the secondary transport of sodium-dependent transporters, such as the CrT. However, a fairly high blood insulin concentration is required to stimulate this transport. In a clinical study, men were given an intravenous insulin infusion at rates of 5, 30, 50, or 105 mU/m^2/minute [427]. The subjects also ingested 5 g creatine, and an additional 7.4 g of creatine was administered by a nasogastric tube 75 minutes later. The four different insulin infusion rates resulted in serum insulin concentrations of 12, 56, 109, and 199 mU/L, respectively. Only these last two concentrations were found to be high enough to improve creatine absorption by the muscles. The amounts of carbohydrate used in other studies were therefore quite high (around 95 g glucose), which makes it less convenient in practice. In comparison, a mixed meal consisting of 75 g starch, 37 g protein, and 17 g fat leads to a peak in the plasma insulin concentration of only 44 mU/L [81]. This would simply not be sufficient to enhance creatine accumulation in muscle tissue.

Since amino acids also induce an insulin response, researchers attempted to replace some of the sugar required for the increased accumulation with whey protein hydrolysate, leucine and phenylalanine [357]. A combination of 57 g glucose, 14 g whey protein hydrolysate, 7 g leucine and 7 g phenylalanine was found to be as effective as 95 g dextrose in enhancing creatine retention.

The form and amount of administration affect the plasma concentration curve [195]. Administration of creatine dissolved in a solution shows a rapid peak in the plasma concentration curve 0.5–1 hour after ingestion. Subsequently, a rapid decrease in the plasma concentration sets in. When a similar amount of creatine is administered as part of a steak, the peak in the plasma concentration curve is delayed ($\sim$1.5 hour after ingestion) and is less high. The subsequent decrease is also more gradual compared to creatine dissolved in a solution. However, the area under the plasma concentration curve is similar between the two.

However, the same researchers demonstrated a smaller area under the plasma concentration curve when an equal amount of creatine was taken as a tablet or suspension. This might be indicative of a lower bioavailability with these dosage forms, due to incomplete absorption from the gastrointestinal tract. Creatine dissolved in a solution is likely to be completely absorbed by the gastrointestinal tract, as a study was unable to find creatine or creatinine in the faeces after a week of supplementation (2 g three times a day) [120]. Given these data, creatine dissolved in a solution should be preferred.

Box 8.1

Many different variants of creatine have appeared on the market. All promising an increased effectiveness compared to conventional creatine monohydrate. One of these variants is creatine ethyl ester. As the name suggests, an ethyl group is attached to the molecule at the carboxyl group. The ethyl ester makes the molecule more lipophilic (less polar), thus making it easier to diffuse across cell membranes and therefore be less dependent on transporter proteins. Indeed, creatine ethyl ester shows an improved permability across intestinal epithelial cells [188]. However, the substance is unstable and degrades very rapidly ($t_{1/2} < 1$ minute). Not surprisingly, a study demonstrated that creatine ethyl ester is less effective than creatine monohydrate in increasing the amount of creatine stored in muscle tissue [423].

To date, no creatine variant has appeared that is more effective than creatine monohydrate. After all, there is little room for improvement over the monohydrate variant, since it already has a very high bioavailability and is cheap to produce.

8.1.3 Metabolism and excretion

The degradation of creatine to creatinine is largely a spontaneous process that isn't accelerated by the intervention of enzymes. Both creatine and creatine phosphate degrade to creatinine. The turnover rate of the latter is slightly higher (2.6 % vs. 1.1 % daily [489]). Creatinine then diffuses out of the tissues and enters the circulation. Subsequently, it's excreted in the urine by the kidneys. As mentioned in Section 8.1.1, a 70 kg adult male between the age of 18 and 29 excretes about 1.7 of creatinine daily (23.6 mg/kg daily) [426].

There's no net creatine or creatinine secretion by the intestines in its lumen, since no creatine or creatinine was found in the faeces after supplementation with creatine for a week [120].

8.2 Mechanism of action

Creatine owes its efficacy to the direct effect it has on the phosphagen energy system (see Section 2.2). It increases the amount of creatine and creatine phosphate in muscle cells,

which in turn can facilitate a larger flux catalyzed by creatine kinase (see Reaction 8.1).

$$PCr^{2-} + ADP^{3-} + H^{+} \leftrightarrow Cr + ATP^{4-} \tag{8.1}$$

This positive effect on cellular energetics results in various effects. A meta-analysis concluded that creatine improved the performed number of repetitions, weight (kg), and generated force (N), power (W) and total amount of work (J) during short ($\leq$30 seconds) high-intensity exercise [63]. It's often argued that the increase in power and number of repetitions will lead to improved training adaptations, which in turn improves strength performance. This would then also lead to an increase in muscle hypertrophy. A double-blind, placebo-controlled trial shows an increase in the cross-sectional area of muscle fibers as a result of creatine supplementation combined with resistance exercise [466]. The subjects were familiar with resistance exercise and had an average body mass of 82.1 ($\pm$11.8) and 82.9 ($\pm$14.3) kg in the creatine and placebo group, respectively. At rest, creatine supplementation has no effect on muscle protein synthesis or breakdown, and as such it needs to be combined with resistance exercise in order to exert an effect [285].

8.2.1 Temporal and spatial energy buffer

Creatine supplementation increases the creatine phosphate (PCr) concentration in muscle fibers [196]. This increases the capacity of PCr to function as a temporal energy buffer. During muscle contraction, adenosine triphosphate (ATP) is used in order to contract the muscle fibers, as described in Section 1.3. PCr is a substrate for the CK reaction (see Reaction 8.1), so that a decrease in ATP can be compensated for by transferring the high-energy phosphate group of PCr to adenosine diphosphate (ADP).

Additionally, creatine can function as a spatial energy buffer. The energy requirements of muscle fibers during contraction are located almost entirely at the myofibrils in the cytosol. However, the oxidative energy system generates energy in a different cellular location: the mitochondria. Direct transport out of the mitochondria is relatively slow, as such, a large part of the energy is exchanged in the form of PCr (creatine phosphate shuttle system, see Section 2.2).

The formation of ATP from PCr and ADP als compensates for a decrease in the pH value (see Reaction 8.1) that originates from the protons generated during hydrolysis of ATP by the myosin ATPases and glycolysis.

8.2.2 Hyperhydration (water retention)

Creatine is an osmotically active substance (for a description of osmosis see Box 11.1). Since a rapid increase in body mass is observed after a loading phase of creatine [312], it's generally believed this increase is the result of water retention. An increased creatine concentration in muscle fibers is believed to cause an increased osmolality, resulting in the attraction of water by the cells. Indeed, an increase in the intracellular fluid compartment of muscle fibers has been shown after a loading phase of creatine using magnetic resonance (MR) imaging [390]. Using multi-frequency bioelectrical impedance analysis, an increase in the intracellular fluid compartment, but not in the extracellular fluid compartment, could be observed after three days of creatine supplementation [505]. A study using dideuterium and sodium bromide dilution analysis (see Box 8.2) to measure total body, intracellular and extracellular fluid, came with some unexpected results [362]. Not too

surprising, they found an increase in total body fluid both 7 days and 28 days after initiation of creatine supplementation. However, the fluid distribution between the compartments remained unaltered. As such, to date there's still some uncertainty about how creatine supplementation affects fluid distribution. It should be noted that only the MR imaging study looked at muscle tissue per se rather than the whole body. The multi-frequency bioelectrical impedance analysis and isotope dilution analysis appear in direct conflict with each other; after all, they both measure fluid of the entire body. The isotope dilution analysis is the most reliable of the two and should therefore be taken as the most likely correct one.

The increase in cell volume as a result of creatine supplementation might also contribute to an increased protein synthesis and decreased protein breakdown rate. Some research suggests that the cellular hydration status affects these processes [200, 273]. Briefly, cell swelling would function as an anabolic signal, whereas cell shrinking would function as a catabolic signal.

Box 8.2

Total body water can be estimated using isotopes such as dideuterium oxide (also known as 'heavy water') [407]. A solution containing the isotope is administered to a test subject. The isotope then spreads pretty much evenly throughout the total body fluid. This dilutes the concentration of the isotope. By subsequently determining the concentration of the isotope from a blood sample, it can be calculated how large the total body fluid compartment is. The following formula can be used for this:

$$V_2 = \frac{C_1 V_1 - C_u V_u}{C_2}. \tag{8.2}$$

V_2 is the volume of water in which the isotope will be completely dissolved at equilibrium (i.e. total body water), C_1 is the concentration of the administered dideuterium oxide, V_1 the volume of the dideuterium oxide solution to be administered (standardized to 37°C), C_u the dideuterium oxide concentration excreted in fluid before equilibrium is reached and V_u the volume of fluid excreted before equilibrium is reached.

After a few hours (two to six), when equilibrium has been reached, the urine loss is determined and a blood sample is taken. The amount of dideuterium oxide in the samples is determined with a scintillation meter. Finally, the total body water can be calculated.

This method can also be used to estimate the amount of fat free mass. Fat free mass consists of approximately 73.2 % fluid (and fat mass contains no fluid). Dividing the measured total body water by 0.732 gives an approximation of the amount of fat free mass.

8.2.3 Satellite cells

A 2006 double-blind, placebo-controlled trial demonstrates that creatine supplementation in combination with resistance exercise enhances the training-induced increase in the number of satellite cells and the concentration of myonuclei [337]. Subjects received 6 g of creatine monohydrate four times a day for 7 days, followed by 7 g once a day for another 15 weeks. Resistance exercise was performed three times a week during the entire trial (16 weeks). Muscle biopsies were taken at four points in time, namely at week 0, week 4, week 8, and week 16. The results of the creatine and placebo group are described in Table 8.1.

An increase in the number of satellite cells per muscle fiber was significant at weeks

4 and 8 for both the creatine group and the placebo group. However, the value was significantly greater in the creatine group compared to the placebo group. Nevertheless, it should be emphasized that at week 16 the number of satellite cells per muscle fiber no longer significantly differed from week 0 in the creatine group, nor was there any significant difference between the two groups.

Furthermore, the relative number of satellite cells was significantly greater at weeks 4 and 8 in the creatine group compared to the placebo group, as was the number of myonuclei per muscle fiber at week 4. And although the mean muscle fiber area did not differ significantly between the two groups at any point in time, there was a significant difference compared to week 0 in the creatine group compared to the other points in time. Because of the large standard deviation and small sample size, there's a considerable chance of a false negative result.

Nevertheless, it's unknown to what extent this mechanism contributes to the ergogenic effects of creatine supplementation. Moreover, it's uncertain to what extent satellite cells contribute to muscle hypertrophy, as discussed in Section 1.5. Future research could clarify this role, as well as its contribution to the ergogenic effect of creatine supplementation.

	Week 0	**Week 4**	**Week 8**	**Week 16**
Number of SC per muscle fiber				
Creatine group	0.11 ± 0.03	$0.23 \pm 0.10^{**a}$	$0.21 \pm 0.07^{**b}$	0.14 ± 0.03
Placebo group	0.10 ± 0.01	$0.13 \pm 0.03^{*}$	$0.14 \pm 0.03^{*}$	$0.13 \pm 0.03^{*}$
Relative number of SC (%)				
Creatine group	5.3 ± 1.3	$9.4 \pm 3.0^{**a}$	$9.8 \pm 2.7^{**b}$	6.1 ± 1.3
Placebo group	4.8 ± 0.6	$6.0 \pm 0.9^{*}$	$6.7 \pm 1.2^{*}$	5.4 ± 1.2
Number of myonuclei/muscle fiber				
Creatine group	1.90 ± 0.23	$2.21 \pm 0.13^{**b}$	$2.13 \pm 0.24^{*}$	$2.13 \pm 0.17^{*}$
Placebo group	1.98 ± 0.18	1.94 ± 0.21	2.06 ± 0.35	2.16 ± 0.30
Mean muscle fiber area				
Creatine group	5268 ± 646	$5983 \pm 849^{*}$	$6003 \pm 1002^{*}$	$6148 \pm 969^{*}$
Placebo group	5052 ± 450	$5752 \pm 765^{*}$	5567 ± 591	5635 ± 648

Table 8.1: Number of satellite cells (SC) per muscle fiber, relative number of SC ($\frac{\text{SC}}{\text{SC+myonuclei}} \cdot 100\%$), number of myonuclei per muscle fiber, and the mean muscle fiber area (μm^2) during a 16-week trial by Olsen et al. [337]. Values are given as means $\pm$ SD. * Significantly different compared to week 0 ($P < 0.05$). ** Significantly different compared to week 0 ($P < 0.01$). ^{a} Significantly different compared to the placebo group ($P < 0.01$). ^{b} Significantly different compared to the placebo group ($P < 0.05$).

8.2.4 S-adenosylmethionine (SAM)-sparing effect

As described in Section 8.1.1, the biosynthesis of creatine requires a methyl group from SAM. It's estimated that about 40 % of SAM is used for creatine biosynthesis [69]. Creatine supplementation inhibits the body's own production of creatine, which means that more SAM will be available for other processes. SAM functions as a methyl donor for various reactions, including homocysteine remethylation. Creatine supplementation indeed results in a slight decrease in the plasma homocysteine concentration [263]. This seemingly confirms the thought that creatine supplementation has a SAM-sparing effect, ensuring more SAM is available for other methylation reactions.

For the time being, it's completely unclear what role this SAM-sparing effect has in regard to the ergogenic effects of creatine.

8.2.5 Modulation of gene expression

Creatine supplementation affects the expression of various genes. Some of these that are involved in muscle hypertrophy are highlighted in Table 8.2.

An increase in the expression of all three myosin heavy chain (MHC) isoforms (discussed in Section 1.4) was observed in a study in which creatine was administered for 12 weeks [480]. The measurements were performed three hours after training. An increase in total protein of the MHC1β- and MHC2a-isoform, but not the MHC2x-isoform, was also observed. Total protein of the MHC2x-isoform decreased. The shift of MHC2x to MHC2a is a normal adaptation that occurs with resistance exercise [283]. Creatine supplementation thus appears to enhance this adaptation.

Another study, which also looked at mRNA expression of MHC1β and MHC2a, did this at several points in time [119]. A 5-day loading phase was used, after which the measurements were made at rest, immediately post-workout, 24 hours post-workout, and 72 hours post-workout. Interestingly, the researchers found an increase in MHCβ mRNA at rest, but not post-workout. This suggests that creatine may stimulate expression of this isoform independent of exercise. They did find an increase in MHC2a mRNA immediately post-workout, but not at rest nor at 24 and 72 hours post-workout. Unfortunately, only mRNA expression was measured and not protein.

8.3 Clinical results

Since the publication of Harris et al. in 1992, an enormous number of studies have been conducted to investigate the effects of creatine in combination with exercise. The large number of studies is more than enough to fill an entire book with, let alone part of a chapter.

Table 8.3 provides a brief summary of the effect on muscle strength and endurance based on two review studies by Dempsey et al. [122] and Rawson and Volek [373]. Rawson and Volek found, on average, an 8 % greater increase in muscle strength (1-, 3-, and 10-RM) compared to placebo when looking at the average of 22 studies. They also found an increase in the maximum number of repetitions that was 14 % higher than those receiving a placebo. However, the authors note that there's a very large interindividual variability. The results can therefore vary drastically from one person to the next: some might hardly notice any effect, while others can achieve strength gains that are vastly higher than the percentages just mentioned. This variability might be attributed to the degree to which creatine supplementation is able to increase intramuscular creatine stores. In some people, especially in whom the creatine concentration normally is quite high to begin with, this increase is small. As previously discussed in Section 3.2, concomitant intake with carbohydrates and protein can improve creatine accumulation in the muscles. This could be a useful strategy for so-called non-responders (people for whom creatine supplementation doesn't seem to have an effect on performance). Rawson and Volek also report that the effect of creatine supplementation is larger in untrained compared to trained individuals [373]. However, a meta-analysis by Branch found no difference between trained and untrained subjects, neither on body composition nor performance [63]. If there were a difference, it would be difficult to quantify this properly, partly because the studies use widely different criteria for the classification of trained and untrained individuals (or

Variable	Timespan	Effect
MHC1β mRNA	12 weeks	$\uparrow$ (3 h post-workout) [480]
	5 days	$\uparrow$ (at rest) [119]
		$\leftrightarrow$ (immediately, 24 h and 72 h post-workout) [119]
MHC2a mRNA	12 weeks	$\uparrow$ (3 h post-workout) [480]
	5 days	$\uparrow$ (immediately post-workout) [119]
	5 days	$\leftrightarrow$ (at rest, 24 h and 72 h post-workout) [119]
MHC2x mRNA	12 weeks	$\uparrow$ (3 h post-workout) [480]
MHC1 protein	12 weeks	$\uparrow$ (3 h post-workout) [480]
MHC2a protein	12 weeks	$\uparrow$ (3 h post-workout) [480]
MHC2x protein	12 weeks	$\downarrow$ (3 h post-workout) [480]
IGF-1 mRNA	5 days	$\uparrow$ (at rest) [121]
		$\leftrightarrow$ (3 h and 24 h post-workout) [121]
IGF-2 mRNA	5 days	$\leftrightarrow$ (3 h and 24 h post-workout and at rest) [121]
GLUT4 mRNA	5 days	$\uparrow$ (at rest) [119]
	5 days	$\leftrightarrow$ (immediately, 24 h and 72 h post-workout) [119]
Myostatin mRNA	5 days	$\leftrightarrow$ (at rest, immediately, 24 h and 72 h post-workout) [119]
MAFbx mRNA	5 days	$\leftrightarrow$ (at rest, immediately, 24 h and 72 h post-workout) [119]
S6K1-P	5 days	$\leftrightarrow$ (3 h and 24 h post-workout and at rest) [121]
	5 days	$\leftrightarrow$ (at rest, immediately, 24 h and 72 h post-workout) [119]
4E-BP1-P	5 days	$\uparrow$ (24 h post-workout) [121]
	5 days	$\leftrightarrow$ (3 h post-workout and at rest) [121]
	5 days	$\downarrow$ (24 h post-workout) [119]
	5 days	$\leftrightarrow$ (at rest, immediately and 72 h post-workout) [119]
ERK1/2-P	5 days	$\leftrightarrow$ (at rest, immediately, 24 h and 72 h post-workout) [119]
Akt-P (Thr308)	5 days	$\downarrow$ (at rest) [119]
	5 days	$\leftrightarrow$ (immediately, 24 h and 72 h post-workout) [119]
Akt-P (Ser473)	5 days	$\leftrightarrow$ (at rest, immediately, 24 h and 72 h post-workout) [119]

Table 8.2: Effects of creatine supplementation on gene expression and phosphorylation state of several proteins.

simply provide too little information about this). In any case, both trained and untrained individuals appear to benefit from creatine supplementation.

The meta-analysis by Dempsey et al. [122] found a significant increase in the 1- and 3-RM bench press and the 1-RM squat (see Table 8.3). No significant improvement was found in 1-RM flexion. However, the data for the 1-RM arm flexion was derived from only three studies, two of which were performed in the elderly. The other study had young men with resistance exercise experience as test subjects. They did find a significant improvement of 5.1 kg compared to placebo in 1-RM arm flexion [38]. Another study, published years after the meta-analysis, also demonstrated more hypertrophy of the arm flexor after creatine supplementation in combination with resistance exercise compared to placebo [79].

There is currently some uncertainty as to whether creatine supplementation is as effective for women as it is for men. While the study by Harris et al. [196] didn't find any differences between men and women in intramuscular creatine accumulation, the number of subjects was small (five women and twelve men). A creatine loading phase showed a trend ($P = 0.052$) for a greater increase in lean body mass in men than women [312]—even

Outcome measure	Finding	Remarks
1- & 3-RM bench press	↑ 6.85 kg (95 % CI = 5.24, 8.47 kg; $n = 143$)	
1-RM arm flexion	↔ 1.53 kg (95 % CI = -1.07, 4.13 kg; $n = 60$)	With subjects older than 60 yr excluded the effect is significant (↑ 5.1 kg).
1-RM squat	↑ 9.76 kg (95 % CI = 3.37, 16.15 kg; $n = 74$)	
1-, 3- & 10-RM muscle strength	↑ 8 % ($n = 443$)	
Number of repetitions*	↑ 14 % ($n = 152$)	

Table 8.3: Effects of creatine supplementation on outcome measures of strength. Abbreviations: CI, confidence interval; RM, repetition maximum. * Defined as the maximal repetitions at a given percent of maximal strength. Data taken from Dempsey et al. [122] and Rawson and Volek [373].

when this increase was expressed in terms of total fat free mass, this trend was maintained. Assuming that the short-term lean mass gain is a result of creatine's osmotic effect, this might indicate that creatine accumulation is less in women than in men.

In line with these results, another study found no increase in lean body mass after six weeks of resistance exercise in combination with creatine supplementation in women, while it did demonstrate an increase in men [93]. However, no muscle biopsies were taken to assess the intramuscular creatine concentration. As such, it remains unclear whether this difference between both sexes is the result of less creatine accumulation in women or not. Additionally, an absolute increase, but not a relative increase, of arm muscle thickness was found in men. Furthermore, there were neither absolute nor relative differences between the sexes for thigh muscle thickness. Remarkably, a greater relative increase in bench press strength was found in women compared to men. For the leg press, the absolute gain in strength was greater for the men (with no difference in relative strength gain). Also, there doesn't appear to be any differences in the effect of creatine supplementation on anaerobic performance between men and women [439].

Other research showed that creatine supplementation in combination with resistance exercise did reduce leucine oxidation and leucine appearance in the circulation (which can be considered a surrogate for protein breakdown) in men, but this effect didn't occur in women [343]. Nevertheless, the study found no differences in mixed muscle protein synthesis.

Taken together, creatine supplementation might have a less positive effect on body composition in women than in men, but otherwise it appears to be just as effective for strength and anaerobic performances as in men.

Although many studies use a loading phase, it's not necessarily required to saturate the muscles with creatine. Even with dosages as low as 3 g daily, the same level of saturation can be achieved [229]. In that case, however, it will take longer before the muscles will finally be saturated—about four weeks. A loading phase thus ensures a faster saturation of the muscles. Moreover, there are indications that the effect of creatine decreases after about two or three months [123]. For example, the increased creatine concentration in muscles appears to decrease again after that period of time. Consequently, it might be advisable to saturate the muscles as soon as possible.

Finally, there is some concern that caffeine intake might counteract the ergogenic effect of creatine. This concern stems from a 1996 paper by researchers at the Catholic University of Leuven [461]. In this study, the effect of creatine, and the combination of creatine and caffeine on a physical test, was compared. The physical test consisted of knee extensions in order to determine dynamic muscle torque. The creatine phosphate concentration in the muscles was also examined. The creatine phosphate concentration increased just as much after supplementation with creatine alone as with creatine and caffeine combined. What was remarkable, however, was that dynamic muscle torque did increase significantly after creatine supplementation, but not after supplementing it together with caffeine.

But what caused this? A few years later, other researchers, also from the Catholic University of Leuven, came up with a possible answer [208]. Creatine and caffeine apparently would have an opposing effect on the relaxation time of muscles (the time required for the calcium concentration in the cytosol to return to the resting level). Creatine shortens the relaxation time [457] and the combination with caffeine would negate this effect. The shortened relaxation time was held responsible by the researchers for the ergogenic effect of creatine. As such, if caffeine could negate this effect it would then also negate the ergogenic effect. What the study showed, however, was that this opposing effect on relaxation time only occurred when caffeine was taken up to 20 hours before the test, but not when taken an hour before the test. Also in that earlier study, in which the effect on dynamic muscle torque was negated by caffeine, subjects hadn't taken caffeine for at least twenty hours after having had high doses of caffeine for three days. Given that symptoms of caffeine withdrawal occur within 12–24 hours [247], it seems more likely that the negative effect these studies found has more to do with caffeine withdrawal than anything else.

8.4 Safety

The scientific consensus is that creatine monohydrate supplementation is safe in healthy individuals [72, 238, 185]. However, some claims have been made in the literature that would cast a shadow on creatine's safety profile. There have been concerns about the kidneys, liver, gastrointestinal tract, cardiovascular system, and even cytotoxicity (being toxic to cells). However, these concerns are based on anecdotal and theoretical evidence. To date, there is no indication that these concerns are justified.

A 1998 case report describes renal dysfunction in an individual supplementing creatine [364]. Not much later (1999), another case report was published, this time describing a case of acute interstitial nephritis (inflammation of the kidneys) in an individual supplementing creatine [264]. Additionally, it's known that creatine supplementation leads to an increase in serum creatinine levels. These levels are commonly used to get an idea of renal function (see Box 8.3), an increase of which might indicate impaired renal function. Both cases have plagued creatine's safety profile for many years. However, it's very likely that creatine had nothing to do with the nephrotoxicity observed in both of these cases. The patient described in the first case had been suffering from glumerosclerosis for several years. This is a condition of the kidneys in which the glomerulus hardens as a result of the formation of connective tissue. In addition, the patient was also taking cyclosporine—a drug known to be nephrotoxic [324]. The patient described in the second case presented with a clinical picture that also closely resembles that of NSAID use. Finally, future controlled research has never been able to demonstrate any nephrotoxicity [360, 386, 302, 268, 182, 327, 184, 79, 288].

Additionally, creatine has no effect on blood pressure (which could otherwise perhaps impact the kidneys) [312, 467, 475, 18, 351].

Box 8.3

Creatine is practically not actively metabolized, so the degradation of creatine into creatinine is fairly constant. In addition, creatinine is completely filtered by the kidneys and is not metabolized any further. This makes creatinine a good candidate as an estimator of renal function (glomerular filtration rate). Normally, when creatinine increases in the serum, it's because its clearance decreases. This means that the kidneys are less able to excrete creatinine, which means a reduced kidney function. Creatine supplementation also leads to an increase in the serum creatinine concentration. However, this is not the result of reduced clearance, but simply caused by an increase in the amount of creatine stored in the body. Thus, there's also an increase in the amount of creatine that's degraded to creatinine per unit of time. In other words: more creatinine is produced, whilst clearance remaince unaltered. The same is seen in individuals with a large amount of muscle mass (since that's the primary site of creatine storage). The slightly increased serum creatinine concentration observed with creatine supplementation is therefore not an indication of impaired renal function.

There is little reason to suspect hepatotoxicity (liver toxicity) with creatine supplementation. A few studies have looked at blood markers that can be indicative of liver damage [251, 386, 302, 268, 92]. None of them showed any abnormalities. To date there is no clinical evidence available that demonstrates hepatotoxicity as a result of creatine supplementation.

Most studies show no change in serum lipids with creatine supplementation [465, 406, 186, 115]. However, one study did observe a change in serum lipids [137]. The study was double-blind and randomized. A significant decrease in total cholesterol (5–6 %), triglycerides (23 %) and VLDL cholesterol (22 %) was observed in the creatine group. The participants initially had a high total cholesterol concentration (>200 mg/dL, i.e. a state of hypercholesterolemia), which might explain why this study found differences whereas other studies didn't. In addition, a trend ($P = 0.051$) towards a lower blood glucose level was found in the creatine group. The authors speculate that this might be indicative of improved insulin sensitivity—something that could also have a beneficial effect on serum lipids. Thus, it's possible that creatine supplementation might have a beneficial effect on serum lipids in certain groups of individuals, however, more research is needed.

Creatine in high doses ($>$ 5 g daily) can lead to gastrointestinal complaints [341]. This is probably caused by creatine's osmotic effect. In addition, creatine is sometimes taken in conjunction with other substances that could possibly lead to complaints, thus falsely designating creatine as the culprit. With low dosages ($\leq$5 g daily) most should not experience any problems. Else, spreading the dosage over multiple smaller doses during the day would be a viable strategy.

Finally, creatine gets metabolized to sarcosine to a tiny extent. This is a reaction catalyzed by creatinase. Subsequent metabolism of sarcosine can then lead to production of methylamine and formaldehyde; two substances known to be cytotoxic (harmful to the cell). A small clinical study shows an increased urinary excretion of methylamine and formaldehyde after fourteen days of creatine supplementation (21 g daily) [361]. Despite the sharp increase, the excreted amount of methylamine was still below the upper limit set for healthy individuals. No such limit is known for formaldehyde, hence there's little

to be said about that. In any case, there's no data to date that indicates this increase in methylamine and formaldehyde is harmful.

Likewise, there is some concern that creatine might be associated with cancer, as it's postulated that it would be facilitated by the formation of carcinogenic heterocyclic amines (HCAs). However, a small-scale study that examined the production of a couple of HCAs couldn't find an increase in any of these as a result of creatine supplementation [349]. It should be noted that clinical research in this direction is extremely sparse, despite the fact that creatine—in general—is very well researched.

8.5 Conclusion

Creatine plays a fundamental role in energy metabolism of the muscular system. It's present in a habitual diet that includes meat and the body is also able to synthesize creatine itself. Creatine supplementation is able to increase the intramuscular creatine concentration above its normal levels. It's almost completely absorbed by transporters in the intestinal wall. Subsequently, creatine easily finds its way into muscle cells—a process that might be enhanced by insulin. Combining creatine with an insulinogenic meal could therefore improve creatine accumulation in muscle tissue. Creatine is practically not actively metabolized by the body. Instead, it spontaneously degrades to creatinine. Creatinine is excreted in the urine by the kidneys.

The increased creatine concentration in the muscle cells as a result of supplementation has a positive effect on cellular energetics. This is the basis for the ergogenic effects of creatine, such as an improvement in muscle strength and endurance during intensive exercise. Unlike many other dietary supplements, the effectiveness of creatine has been proven beyond doubt.

Typically, a loading phase of a few days with a high creatine intake of 20 g daily is practiced. The dose can be divided into four smaller doses of 5 g. After this loading phase, a maintenance phase of 3–5 g daily can be used. Taking a higher dose at one time is inadvisable, because of the risk of gastrointestinal discomfort and probable incomplete absorption at higher doses.

The long-term effectiveness of creatine supplementation remains to be proven. There are some indications that the effectiveness of creatine decreases after about two or three months of supplementation. For that reason, it might be advisable to include a rest phase, during which no creatine is supplemented, of four to six weeks every two to three months.

Finally, creatine has an excellent safety profile and there is a consensus that creatine supplementation is completely safe in healthy individuals.

9. Protein supplements

9.1 Introduction

Proteins constitute an important part of the diet. The proteins you eat ensure that the body can maintain many of its functions. The building blocks of proteins are amino acids. In total, twenty amino acids are used for protein synthesis in the human body. Eleven of these amino acids can be synthesized by the body *de novo* (can be made 'from scratch'). However, the other nine are indispensable in the diet, because they cannot be synthesized *de novo*. These nine amino acids are therefore also called the essential amino acids. The human body needs amino acids for many processes. For example, some amino acids can function as a neurotransmitter (e.g. glutamate) or form the precursor for the synthesis of a neurotransmitter (e.g. adrenaline) or a hormone (e.g. thyroxine [T_3]). In addition, they are, of course, necessary for the synthesis of new proteins. Moreover, amino acids can function as a source of energy for the body.

Meat, fish, eggs, and dairy products in particular contribute a great deal to the daily protein intake. For vegetarians and vegans, other foods are often an important source of protein, such as nuts, mushrooms, legumes and grain products. The Dutch Health Council recommends a protein intake of 0.8 g/kg bw per day for adults [161]. For most people this amounts to a daily protein intake of about 50 to 70 g. This amount is easily obtained from the diet. However, for athletes who seek to increase their muscle mass, a higher amount is required. It has been estimated that the requirement for this population can increase up to 3.1 g/kg lean body mass per day under certain conditions [205]. This can make it challenging to obtain sufficient protein from the diet without using protein supplements. Many athletes therefore use these to supplement their diet.

There are various types of protein supplements, most of which consist of whey protein

or casein protein. Both are protein fractions derived from milk. About one fifth of milk protein consists of whey and four fifths of casein [414]. There are also supplements available that contain pea protein, egg protein, soy protein, rice protein, beef protein, hemp protein, etc. Whey and casein protein supplements, however, are the most popular. Additionally, most research into protein supplements has used these two types. This chapter will therefore mainly focus on whey and casein supplements.

Since amino acids are the building blocks of the proteins that we synthesize, and therefore also the muscle proteins myosin and actin, it shouldn't come as a surprise that protein intake is important for optimal muscle growth. On the one hand, sufficient aminoacyl-tRNAs must be available for muscle protein synthesis, and on the other hand, amino acid availability plays a key role in the regulation of muscle protein synthesis. This last point in particular is the reason why such large amounts of protein are required to optimize muscle growth. After all, the amount of muscle protein that's built during a period of muscle growth is only a tiny fraction of the total amount of protein that is consumed from the diet during this period. Central to this is mTOR signaling, as discussed in Section 4.2. Although some years ago it was thought that this regulation was primarily controlled by the essential amino acid leucine, several other amino acids turn out to also be closely involved. Either indirectly by stimulating cellular leucine uptake, or directly by affecting mTORC1 activity [167]. This emphasizes the importance of protein intake with a complete amino acid profile.

9.1.1 Absorption

Once protein enters the stomach, it's exposed to its acidic environment. The stomach acid causes the proteins to rapidly lose their spatial structure. This process is called denaturation. As a result, the proteins become more accessible to enzymes that can break the peptide bonds between the amino acids they consist of. In the stomach this function is fulfilled by the enzyme pepsin. Pepsin is an endopeptidase, which means that it breaks the peptide bonds of the non-terminal amino acids. In other words: it cleaves the protein strands into two smaller peptides. The resulting mixture of polypeptides and oligopeptides (short peptide chains) is then further processed in the small intestine by other peptidases that are secreted by the pancreas, as well as membrane-bound peptidases located on the enterocytes. These hydrolyze the peptide chains further to free amino acids, di- and tripeptides, which are then absorbed by the intestinal mucosa.

The absorption of free amino acids by the enterocytes is performed both by active transport and facilitated diffusion. The transporters recognize certain classes of amino acids rather than that they're specific to certain amino acids. Because of this, excessive intake of one amino acid has the potential to limit the absorption of other amino acids that make use of the same transporter(s). These transporters are subdivided into five different transport systems [66]:

1. the neutral system (with a preference for uncharged amino acids);
2. the basic system (with a preference for positively charged amino acids and cystine);
3. the acidic system (with a preference for negatively charged amino acids);
4. het iminoglycine (with a preference for proline, hydroxyproline, and glycine); and
5. the β-amino acid system (with a preference for taurine and β-amino acids).

Most of these transporters are Na^+- or Cl^--dependent.

The absorption of di- and tripeptides across the apical membrane of enterocytes is facilitated by peptide transporter 1 (PEPT1) [111]. This transport is dependent on the proton

gradient maintained by a sodium proton antiporter. In turn, this sodium proton antiporter relies on sodium potassium pumps that get rid of sodium across the basolateral membrane. Once arrived in the enterocyte, the di- and tripeptides undergo further processing into free amino acids by peptidases. After this, these amino acids, together with the other absorbed free amino acids, leave the cell again by crossing the basolateral membrane. This way they end up in the portal vein, thus passing through the liver and finally ending up in the general blood circulation.

However, a large portion of the dietary amino acids are already consumed by the intestines and liver to fulfill their energy and protein synthesis demands [440, 456]. The intestinal protein synthesis therefore rises sharply after eating a protein-rich meal. During fasting, however, the intestines ensure that the rest of the body is still supplied with amino acids. During that period of time, net protein breakdown occurs in the intestinal cells. The liver can also compensate for a shortage of a certain non-essential amino acid from the surplus of another amino acid. For example, transamination (transfer of the NH_2 group) of glutamic acid to pyruvic acid can take place to compensate for an alanine deficiency. The intestines and liver thus play a central role in the regulation of the amino acid supply to the rest of the body. The ratio of amino acids that appear in the circulation, and the speed with which this happens, is therefore not an exact reflection of what's being absorbed by the intestinal cells.

The rate of absorption by the intestinal cells depends on the physico-chemical properties and amount of the ingested protein. When protein is consumed as part of a meal, the rate of absorption also depends on what else makes up the meal. Although most dietary protein is part of a complete meal, protein supplements are often taken in isolation by athletes. It's therefore useful to look at the kinetic properties of whey protein and casein protein per se.

Whey protein and casein protein are often reffered to as 'fast' and 'slow' protein, respectively [57]. Whey protein is rapidly absorbed by the intestinal mucosa after ingestion. However, the absorption of casein occurs much slower, probably because the protein forms clots in the acidic environment of the stomach and thereby slows down gastric emptying.

After ingestion of whey protein, a peak in the plasma leucine concentration [57] or total amino acid concentration [77] takes place after approximately 1–1.5 hours. This is also the case in the elderly [346, 347]. The protein is quickly digested and absorbed, so that it's almost completely absorbed after about four hours. When ingesting casein protein, a peak in the plasma leucine concentration occurs about as fast as with ingestion of whey protein [57]. However, the peak is much lower because the absorption proceeds at a slower rate. As a result, casein protein yields a longer-lasting release of amino acids into the circulation than whey protein. However, the peak in the plasma leucine concentration after casein protein ingestion is also lower because it simply contains less leucine than whey protein does. One study combined whey or casein protein with double cream (a greasy type of cream) and maltodextrin to see how it would affect absorption kinetics [190]. The peak total amino acid concentration is well comparable to the results of Calbet & Holst, as depicted in Table 9.1 (note that the peak value by Hall et al. is reported minus the baseline concentration of approximately $\sim$2 mmol/L). This suggests that the addition of a small amount of easily digestible carbohydrates and fat has little to no effect on the absorption of whey and casein protein.

From the circulation, the amino acids reach the interstitium and can then be taken up by the cells. Transport from the circulation into the interstitium appears to be the rate-limiting step rather than the absorption of amino acids across the sarcolemma from

the interstitium [313, 174]. The passage across the capillary walls largely takes place by means of diffusion. This might also play a role in the so-called 'anabolic resistance' that's observed in the elderly (see Box 9.1).

Box 9.1

In the elderly, basic protein metabolism is intact, but the effect of protein intake and exercise on muscle protein synthesis is hampered. This is also called anabolic resistance [382]. It has been hypothesized that the reduced effect on muscle protein synthesis by protein intake would be, at least in part, be caused by reduced amino acid transport across the capillary walls. The endothelial function deteriorates with age. This process also underlies an increased risk of cardiovascular disease with aging [411]. Inducing hyperaemia (increased blood supply) by administering a vasodilator has been shown to be effective in negating this reduced effectiveness of amino acids on muscle protein synthesis in the elderly [128]. Exercising regularly and a healthy diet can contribute to maintaining good endothelial function with aging.

As mentioned earlier, some of the amino acids that are ingested are already used by the intestinal system. The branched-chain amino acids (BCAAs) leucine, isoleucine and valine are largely left untouched, however. After eating a protein-rich meal, the BCAAs constitute more than half of the amino acids in the blood that's leaving the intestines [470], while the BCAAs make up roughly one fifth of the protein ingested. BCAAs are largely taken up by muscle tissue [298]. This is facilitated by the neutral transport system, which prefers uncharged amino acids. After all, leucine, isoleucine, and valine are all uncharged. Specifically, system L (L-type amino acid transporter 1 [LAT1]) and system A (sodium-coupled neutral amino acid transporter 2 [SNAT2]) appear to be involved here [132].

9.1.2 Metabolism and excretion

The proteins from the diet are broken down into free amino acids after which they can be used by the body to synthesize new proteins. In addition, they can serve as an energy source or as a precursor for a neurotransmitter or hormone. Moreover, some amino acids can be used to synthesize other non-essential amino acids. The metabolism of amino acids is thus quite complex and extensive for these reasons.

When we look at the breakdown of amino acids, two elements are central to it: the carbon skeleton and one or several nitrogen groups that an amino acid consists of. The carbon skeleton can be used to fulfill the energy needs of a cell. The carbon skeleton is converted into glucose or a keto body precursor for this purpose. The amino acids that are broken down into glucose precursors (pyruvate, α-ketoglutarate, succinyl-CoA, fumarate and oxaloacetate) are called glucogenic amino acids. The amino acids that are broken down to ketone body precursors (acetyl-CoA and acetoacetate) are called ketogenic amino acids. Some amino acids can form both glucose and ketone body precursors. Ultimately, these precursors end up being used in the mitochondria to obtain energy from.

Box 9.2

To gain some insight into liver function, ALAT and ASAT measurements are often requested on a blood panel. Both enzymes are so-called transaminases and are responsible for the transfer of an α-amino group to an α-keto acid.

Reference	Type	Sample size	T_{max}	C_{max}
Boirie et al. (1997)[a] [57]	30 g whey protein	$n = 6$	80 min	370 μmol/L
Boirie et al. (1997)[a] [57]	43 g casein protein	$n = 6$	40 min	280 μmol/L
Hall et al. (2003)[b, d] [190]	48 g whey protein, 19 g double cream (9 g fat), 20 g maltodextrin	$n = 9$	75 min	2.0 mmol/L
Hall e.a. (2003)[b, d] [190]	48 g casein protein, 23 g double cream (11 g fat), 24 g maltodextrin	$n = 9$	45 min	1.44 mmol/L
Calbet & Holst (2004)[b] [77]	60 g whey protein	$n = 6$	60 min	4.75 mmol/L
Calbet & Holst (2004)[b] [77]	60 g casein protein	$n = 6$	60 min	4.15 mmol/L
Calbet & Holst (2004)[b] [77]	60 g hydrolyzed whey protein	$n = 6$	30 min	4.6 mmol/L
Calbet & Holst (2004)[b] [77]	60 g hydrolyzed casein protein	$n = 6$	20 min	4.75 mmol/L
Pennings et al. (2011)[c] [346]	20 g whey protein	$n = 16$	60 min	2.05 mmol/L
Pennings et al. (2011)[c] [346]	20 g casein protein	$n = 16$	30 min	1.35 mmol/L
Pennings et al. (2011)[c] [346]	20 g hydrolyzed casein protein	$n = 16$	45 min	1.8 mmol/L
Pennings et al. (2012)[c] [347]	10 g whey protein	$n = 11$	45 min	1.75 mmol/L
Pennings et al. (2012)[c] [347]	20 g whey protein	$n = 11$	45 min	2.1 mmol/L
Pennings et al. (2012)[c] [347]	35 g whey protein	$n = 11$	60 min	2.45 mmol/L

Table 9.1: Pharmacokinetic data of various whey and casein protein supplements. T_{max}- and C_{max} value assuming: [a] leucine concentration, [b] total amino acid concentration, [c] total essential amino acid concentration. [d] C_{max} value reported minus the baseline concentration. The baseline concentration in a fasted state is usually $\sim$2 mmol/L.

This yields a new amino acid and a new α-keto acid.

Both transaminases end up in the blood circulation as a result of liver damage. As such, these are used as biomarkers for liver damage. However, the same transaminases are also present in muscle tissue and can be released into the circulation by exercising. A small study showed a large increase in the serum transaminases concentration after a weightlifting session in healthy men [353]. Even seven days later, there was still a significant increase. These findings need to be taken into account when interpreting these blood values, especially in athletes whom participate in resistance exercise.

The body gets rid of the nitrogen groups derived from amino acids by converting these into ammonia, after which they enter the urea cycle. This process takes place in the liver. First, an amino acid is stripped of its α-amino group, after which this group is, in most cases, converted into ammonia. Central to this is the amino acid glutamate. The amino groups of amino acids are transferred onto an α-keto acid which yields glutamate. Subsequently, the amino grouop of glutamate can be removed to form ammonia. Ammonia is toxic to cells and therefore high concentrations of it are not desirable. To cope with this, the cells convert ammonia to the harmless compound urea in a five-step process (the urea cycle). Urea can then easily be excreted by the body.

Important for the transfer of an amino group onto α-ketoglutarate to form glutamate are the transaminases alanine aminotransaminase (ALAT) and aspartate aminotransaminase (ALAT). See also Figure 9.1. The serum concentrations of these transaminases are used in liver disease diagnostics (see Box 9.2).

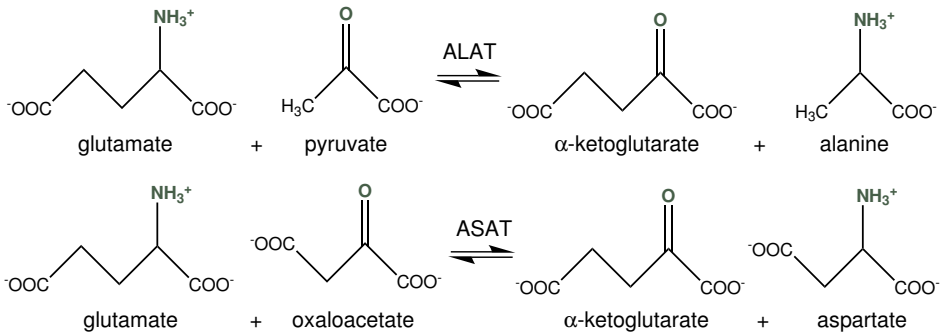


Figure 9.1: The two transaminases ALAT and ASAT are responsible for the transfer of an amino group onto α-ketoglutarate in order to form glutamate. ALAT transfers the amino group of alanine onto it, and ASAT that of aspartate. The reaction is reversible and, as such, alanine and aspartate can also be formed from glutamate by ALAT and ASAT, respectively.

9.2 Mechanism of action

Resistance exercise in combination with an adequate protein intake is known to promote muscle hypertrophy. Both form the foundation for any athlete wishing to increase muscle mass. In 1995, researchers attempted to answer the question whether resistance exercise alone also led to a net deposition of protein in muscle tissue in the hours after an exercise

session [44]. Despite the significant increase in muscle protein turnover, no net protein storage occurred in the three hours after a fasted training session. This is not that surprising; if it actually did lead to a net muscle protein storage, this would have been at the expense of some other tissue. After all, a cell depends on a source of amino acids to incorporate them into new proteins.

The same researchers also examined the effect of intravenous amino acid administration at rest and after resistance exercise [45]. The administration of amino acids caused a net storage of protein in muscle tissue after a training session, and this effect was stronger compared to at rest. Many follow-up studies conducted with protein supplements also show a greater acute increase in protein synthesis after a training session compared to placebo [378].

But what's the underlying mechanism? Protein consists of amino acids, and these amino acids function as building blocks for the body to synthesize new proteins from. Thus, they function as a substrate for protein synthesis and, consequently, the growth of a muscle. However, what's also very important is that they function as signaling molecules to stimulate anabolic signaling. This occurs mainly via activation of the mechanistic target of rapamycin complex 1 (mTORC1). In response to an increased amino acid concentration, mTORC1 is activated (see Section 4.2 for an extensive discussion on mTORC1 regulation). In turn, mTORC1 phosphorylates its two primary substrates eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and ribosomal S6 kinase 1 (S6K1). Both are tightly involved in protein translation. Phosphorylation of these molecules by mTORC1 leads to increased protein synthesis. The activation of mTORC1 by amino acids has been discussed in detail in Section 4.2.3.

9.2.1 Stimulation of mTORC1 signaling

As described in Section 4.2.3, amino acids are important regulators of the mTORC1 pathway. Multiple studies have looked at the effect of resistance exercise in combination with whey protein [148, 228, 147, 252, 249] and casein protein [379] intake on mTORC1 signaling.

A study in young untrained men (18–25 years old) looked at the effect of whey protein isolate (26 g) taken immediately after 3 sets of 12 repetitions of knee extensions [148]. Muscle biopsies were taken 2, 4, and 24 hours after the knee extensions to determine the phosphorylation status of several molecules involved in the mTORC1 pathway. Akt phosphorylation (Ser473) was unaffected. However, there was a significant increase in mTOR (Ser2448), 4E-BP1 (Thr37/46) and S6K1 (Thr389) phosphorylation two hours after the exercise bout compared to the placebo group.

Later, the same researchers came up with a follow-up study consisting not only of a group of young untrained men, but also a group of untrained older men (60–75 years old) [147]. At baseline, a muscle biopsy was taken two hours after performing three sets of eight repetitions of knee extensions. Subsequently, the subjects completed a 12-week resistance exercise program. After these twelve weeks, another muscle biopsy was taken after the subjects performed knee extensions. Comparing the results of the two biopsies helps answering questions with regard to the impact of chronic resistance exercise on mTORC1 pathway sensitivity. This is an important question to answer, since many studies showed a shortened and decreased response in muscle protein synthesis after a workout in trained subjects [378]. The results at baseline were similar to what the researchers had found in earlier research. Akt phosphorylation (Ser473) remained unchanged in both the

young and the elderly. mTORC phosphorylation (Ser2448) increased in both whey groups compared to placebo. However, the increase in 4E-BP1 and S6K1 phosphorylation was only significant in the elderly. The second muscle biopsy, after completion of the resistance exercise program, also showed no changes in Akt phosphorylation. The increase in mTOR phosphorylation that was initially significant in both the young and the elderly was now significant only in the young. The increase that was observed in the young was pretty much the same both before and after completion of the program. Additionally, the sharp increase in S6K1 phosphorylation that had initially been observed in the elderly was now greatly reduced and no longer significant. These results suggest that the anabolic response to exercise and protein intake in the elderly is diminished in the trained state, while this doesn't appear to be the case in the young.

A study from another research group also looked at the effect of protein intake (15 g whey protein isolate) on mTORC1 signaling after exercise in young men in both untrained and trained condition (21 weeks of resistance exercise) [228]. S6K1 phosphorylation (Thr389) was greatly increased in the untrained group that received protein at 1 hour post-exercise, but wasn't anymore after 48 hours. No increase was found after completion of the resistance exercise program. Remarkably, the other substrate of mTORC, 4E-BP1, demonstrated a decrease (although it wasn't significant at $P = 0.06$) in phosphorylation status in the placebo group 1 hour post-workout, which didn't occur in the group receiving protein. This suggests that whey protein is able to inhibit this exercise-induced decrease. When looking at mTOR phosphorylation (Ser2448) per se, a significant increase is seen 1 and 48 hours post-workout. This increase also still occurred after completion of the training program. No significant increases were found in the placebo group.

Other research in untrained subjects showed similar results, although it did show an increase in Akt phosphorylation (Ser473) 1 hour post-workout in combination with ingestion of 10 g or 20 g whey protein hydrolyzate [249]. An increase in Akt phosphorylation (Ser473 and Thr308) was also found by Reitelseder et al. when administering $\sim$20 g whey protein isolate or casein protein post-workout [379]. It's unclear why these studies did find an increase in Akt phosphorylation. A possible explanation might be the low sample sizes in these studies, which makes it harder to detect differences. Reitelseder et al. also found that the effect of whey protein isolate and casein protein on Akt and S6K1 phosphorylation was similar, although the ratio 4E-BP1-P (Thr37/46) to total 4E-BP1 a larger increase in the casein protein group compared to the whey protein isolate group. This appeared to be mainly caused by the increase in total 4E-BP1 in the whey group.

9.3 Clinical results

The effect of protein supplementation, or a high protein diet, on muscle mass and strength is relatively small. Because of this, many studies find no benefit from protein supplementation over placebo due to their small sample sizes. Thus, to answer the question to what extent protein supplementation or a high protein diet ($>$ 1.2 g/kg bw/d) has an effect, a meta-analysis was needed. Based on 22 studies with 680 subjects, this meta-analysis found a significant increase in fat free mass (FFM), muscle fiber cross-sectional area, and 1-RM leg press [88]. The results are listed in table 9.2. FFM increased on average by 0.69 kg in the 22 studies. This increase was similar for both young adults and the elderly. This increase was also similar for trained and untrained young adults. The FFM increase in young adults, which averaged approximately 1 kg after 12 weeks, was apparent even

though the groups already had a protein intake higher than 1.2 g/kg bw/d at baseline. They received, on average, an additional 50 g protein on top of this. Interestingly, the protein supplementation had the largest relative FFM difference compared to the placebo group in the trained young adults. This emphasizes the importance of an adequate protein intake in trained individuals to achieve optimal muscle hypertrophy.

Outcome measure	Finding	Remarks
VVM	$\uparrow$ 0.69 kg (95% CI = 0.47, 0.91 kg)	
VM	$\leftrightarrow -0.11$ kg (95% CI = -0.50, 0.29 kg)	
CSA type 1 muscle fiber	$\uparrow$ 212 μm^2 (95% CI = 109, 315 μm^2)	Young adults only.
CSA type 2 muscle fiber	$\uparrow$ 291 μm^2 (95% CI = 71.7, 510 μm^2)	Young adults only.
1-RM leg press	$\uparrow$ 13.5 kg (95% CI = 6.4, 20.7 kg)	

Table 9.2: Effect of protein supplementation in combination with $\geq$ 6 weeks resistance exercise compared to placebo. Data taken from Cermak et al. [88]. Abbreviations: CI, confidence interval; FFM, fat free mass; FM, fat mass; CSA, cross-sectional area; 1-RM, 1-repetition maximum.

A question that's difficult to answer is what amount of protein is optimal for muscle growth. Some important factors that influence this are energy intake, age, protein source, FFM, and fat mass [74, 205, 322, 354]. It can be argued that a lower energy intake, older age, less complete protein sources, a higher FFM, and a lower fat mass, increase protein requirements for optimal muscle hypertrophy. Among other reasons, these factors make it difficult to draw conclusions from various studies. In addition, you could say the law of diminishing returns applies to protein intake. The higher the protein intake, the smaller the extra effect on muscle mass it yields. This makes it even more difficult to find a statistically significant effect (if there is one) with higher protein intakes. Nevertheless, recommendations have been made in the literature that are considered to be optimal—although these are still speculative to some extent due to the limitations of the available research.

Stuart Phillips and Luc van Loon propose an optimal protein intake for maximizing muscle protein synthesis of 1.3–1.8 g/kg bw/d [355]. They also state that during an energy-restricted diet, the optimal protein intake might rise to 1.8–2.0 g/kg bw/d. It also assumes the protein comes from high-quality proteins (e.g. animal proteins from meat, fish and dairy products). A study among Dutch athletes found that the average protein intake was 1.5 g/kg bw/d in strength athletes [163]. Thus, in general, athletes seem to be close to this range.

Eric Helms et al. examined the literature to postulate an optimale protein intake during a calorie-restricted diet in trained athletes [205]. They arrived at a recommendation of 2.3–3.1 g/kg FFM/d, with a lower body fat percentage and a greater calorie deficit leading to the highest requirement. Since it's plausible that protein requirements increase with the amount of FFM, but not fat mass, they expressed the protein requirements in terms of FFM instead of body weight. Converted to total body weight, their range equates to 2.0–2.7 g/kg

bw/d for an athlete with a fat percentage of 12 %.

Protein quality also plays an important role in optimizing muscle growth. The absorption rate and the amount of essential amino acids present, in particular leucine, are of particular importance here [438, 354]. Leucine plays a prominent role as a regulator of the mTORC1 pathway and thus as a regulator of protein synthesis. In a study in which untrained subjects followed a resistance exercise program, combined with either whey protein or soy protein, those receiving whey protein gained more lean body mass (LBM) than those who received soy protein [468]. Soy protein contains a lot less leucine than whey protein and this might be the reason why whey protein led to a greater LBM increase.

As mentioned, the absorption rate is likely of importance too. One study looked at the effect on mixed muscle protein synthesis after ingestion of whey protein hydrolyzate (rapidly absorbed), soy protein isolate (rapidly absorbed) and micellar casein protein (slowly absorbed) [438]. The amount of each was equated so that they all provided 10 g of essential amino acids. As a result, the amount of leucine was a bit higher in those receiving whey protein than those receiving either soy protein or micellar casein protein (with no difference in leucine contents between the soy protein and casein). As expected, there was a slightly higher increase in mixed muscle protein synthesis post-workout in the whey group compared to the other two groups. In addition, a greater increase in mixed muscle protein synthesis was found in the group receiving soy protein compared to the group receiving casein protein. The area under the plasma leucine concentration curve was smaller after ingestion of casein protein, suggesting that the rate of absorption is indeed the underlying cause of this difference. Something similar is seen when whey protein is compared to rice protein or pea protein. However, taking a larger dose of these qualitatively inferior proteins, in order to obtain a sufficient increase of (particularly) leucine in the circulation (the hypothesized leucine threshold), leads to comparable results [354].

For a long time it has been believed that protein intake immediately post-workout played a crucial role in optimizing muscle hypertrophy. It was proposed that there was a limited amount of time after a workout in which protein intake would have optimal effects, also called the ‘anabolic window’. Protein intake taken immediately before a workout is also considered to exploit the anabolic window. However, a meta-analysis found no effect on muscle hypertrophy using this anabolic window after correction for total protein intake [408]. If there was an effect, it need to be (very) small. A disadvantage of this meta-analysis is that there were only two studies available in which the subjects were trained and the control group was matched for total protein intake. In one of these two studies, a group received 40 g whey protein isolate, 43 g glucose, and 7 g creatine, taken both immediately pre- and post-workout. Another group received the same thing in the morning and the evening instead of pre- and post-workout [107]. The group that took their supplement around their workout showed a significant increase in FFM and cross-sectional area of type 2 muscle fibers after ten weeks of resistance exercise compared to the group that received the supplement in the morning and evening.

The other study, also with trained subjects, was similar in design. One group received 42 g protein and 2 g carbohydrates immediately pre- and post-workout, and the other group received this early in the morning and late in the evening [216]. No differences were found between the two groups after ten weeks of resistance exercise. In summary, it could be stated that, if an anabolic window were to exist, it would probably extend for a longer period of time than immediately pre- and just after (1 hour) a workout and might be of importance for trained individuals.

Since there seem to be no disadvantages, and many people experience it as a practical moment anyways, it's of course a useful strategy to supplement protein post-workout. The optimal dose that can be used for this to maximize muscle protein synthesis appears to be around 40 g whey protein, although it offers only a slight improvement compared to half this amount [292]. For the elderly, however, higher doses appear to be relatively more improtant than for the young [316]. Finally, a greater amount of proteins containing relatively low amounts of leucine is likely to be required. This can be disadvantageous, as a greater intake also implies a greater energy intake, which is not always desirable.

9.4 Safety

Proteins are an essential part of the diet. A normal protein intake is necessary to stay healthy. A deficiency can lead to various pathologies that can be fatal. Although a protein deficit does still occur in developing countries, it hardly occurs in countries such as the Netherlands. The question that remains, especially for athletes, is whether a high protein intake is safe. There is no clear-cut definition for a high protein intake and there is no clear evidence indicating that a high protein intake is harmful to otherwise healthy individuals. However, the Dutch Health Council notes a safe upper limit of intake of 25 % of daily energy intake for adults. For an athlete with an energy intake of 3000 kcal, this translates to 188 g of protein daily. In the United States, the Institute of Medicine (IOM) recommends a range of 10–35 % of daily energy intake for healthy individuals.

The concerns surrounding high protein intake are twofold. On the one hand, there are concerns about the direct harmfulness of large amounts of protein, and on the other hand there are concerns about the harmfulness of large amounts of protein-rich products such as meat and dairy. A discussion of the latter is beyond the scope of this book and this section will therefore be limited to the potential harmfulness of a high protein intake per se.

A high protein intake is supposedly bad for the kidneys. Proteins increase, among other things, the glomerular filtration rate (GFR) and this would eventually have a negative impact on kidney function [296]. While this might be the case in people with pre-existing kidney disease, there is no indication that this also holds true for healthy individuals. The increased GFR appears to be a completely normal physiological adaptation to the increased protein intake. Many athletes have a habitual protein intake that is (very) high, especially strength athletes. Yet there is no indication that there is a higher prevalence of kidney disease among athletes. A study from 2000 found no indication of impaired renal function in a small group of athletes with a high protein intake up to 2.8 g/kg bw/d [359]. In a study by Antonio et al., a high protein intake ranging from 2.6 g/kg bw to 3.3 g/kg bw was combined with heavy resistance exercise for 16 weeks [15]. No effect was found on renal function. Later research, also by Antonio et al., similarly found no effect on kidney function after subjects had been consuming a high protein diet for a year combined with resistance exercise [16].

As described earlier in this chapter, all amino acids contain at least one nitrogen group. The body gets rid of these nitrogen groups by converting them into ammonia. Ammonia is then fed into the urea cycle, which turns it into urea. The produced urea is then concentrated in the urine by the kidneys. A small portion of the generated ammonia is also concentrated in the urine by the kidneys. This increases the amount of fluid required by the body, and thus would confer a risk of dehydration. Approximately 40–60 mL of water is required for the excretion of 1 g urea nitrogen [296]. A gram of animal protein contains about 0.16 g of

nitrogen. If someone were to increase their protein intake by 50 g, coming from animal protein, this would increase fluid requirements by only 320–480 mL. Such amounts are relatively small compared to the amount of fluid lost during intense exercise, for example. Although dehydration does occur in athletes, this is almost without exception caused by the large amounts of fluid lost through the skin by sweating during exercise, especially when performing exercise in high ambient temperatures at high intensity.

There is also some concern about the effect of a high protein intake on the skeleton. For example, a higher protein intake undoubtedly leads to a higher excretion of calcium in the urine [257]. If this calcium were to come from the skeleton, it would have a negative effect on bone mineral density. The reasoning behind this is that the acidifying effect of proteins is buffered by the body by extracting calcium carbonate from the skeleton [36]. However, the extra calcium that is excreted appears to come from increased calcium absorption by the gastrointestinal tract. As such, it doesn't appear to have a net negative effect on the skeleton [257]. In general, cross-sectional studies and prospective randomized trials show no negative effect of protein supplementation on bone mineral density, and possibly even a small positive effect [112].

9.5 Conclusion

Protein is an important part of our diets. The recommended daily intake is 0.8 g/kg bw/d for non-athletes. The amount that is optimal for strength athletes is still fairly speculative, but could be as high as 3.1 g/kg FFM/d in athletes with a low fat percentage on an energy-restricted diet. Protein supplements are very useful as a dietary supplement for athletes to meet this increased protein requirement. The milk protein fractions whey and casein in particular have been well researched in this regard. The time of protein intake is of relatively little importance, as long as it's reasonably spread throughout the day. An optimal dose to maximize muscle protein synthesis post-workout would be around 40 g of whey protein. Lower quality proteins, particularly those low in leucine, likely require a higher dose to achieve the same effect. However, the extra improvement of this dose compared to half this amount, is quite small—which can play a role when there's a limited budget.

The effectiveness of a high protein diet or protein supplementation on increasing FFM or improving strength gains in combination with resistance exercise is well documented in the literature. This seems to become relatively more important in trained athletes. In addition to its effectiveness, the safety of high protein intakes is, as of present, also reasonably well documented in the literature. Although concerns have been raised about high protein intake and renal function and bone mineral density, these concerns are not supported by the literature.

10. Phosphatidic acid

10.1 Introduction

Phosphatidic acid is a phospholipid consisting of a glycerol backbone with two fatty acids and one phosphate group attached to it. The two fatty acids are attached to the first two carbons atoms, with the phosphate group attached to the third carbon atom. It's therefore also called a glycerophospholipid. The fatty acid attached to the first carbon atom is usually a saturated one of sixteen or eighteen carbon atoms in length. The fatty acid attached to the second carbon atom is usually unsaturated one of sixteen, eighteen or twenty carbon atoms in length. However, the fatty acids of soy-derived phosphatidic acid (commonly used in supplements) consists only of fatty acid chains that are sixteen or eighteen carbon atoms in length [367]. Phosphatidic acid is present in cell membranes in low concentrations (see Box 10.1). The molar concentration is less than 5 % of that of phosphatidylcholine, the most common glycerophopholipid found in cell membranes [71].

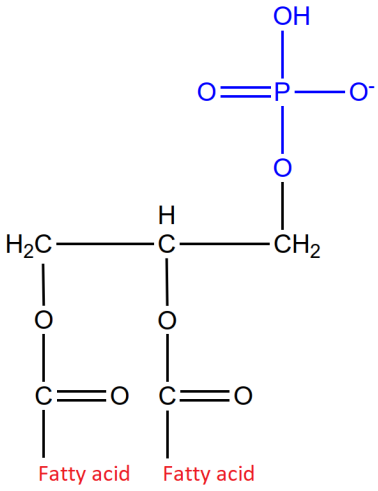


Figure 10.1: Structural formula of phosphatidic acid. Two fatty acids are attached to the first and second carbon atom of glycerol, and a phosphate group (in blue) is attached to the third carbon atom.

Although phosphatidic acid is still relatively unknown as a dietary supplement among athletes, the phospholipid has already attracted quite some attention from researchers [60]. As it goes, phosphatidic acid is tightly involved in activation of mTORC1 (see Section 4.2.4). In 2009, a model was proposed in which phosphatidic acid was the key mediator in activating mTORC1 in response to eccentric muscle contractions [338]. It didn't take long before it caught the attention of researchers in the sports supplements field. In 2012, the first clinical trial examining the effect of phosphatidic acid supplementation on strength, muscle thickness and lean body mass was published [217].

Phosphatidic acid can also be found in the diet, but only in very small quantities (see Table 10.1). The richest phosphatidic acid source in the diet appears to be raw cabbage, with a concentration of half a milligram per gram. Either way, it's pretty hard in practice to get as much phosphatidic acid from the diet as what is used as a dose in clinical trials (several hundred milligrams).

Product	Phosphatidic acid (mg/g)
Cabbage	0.49
Cabbage (cooked)	0.03
Tomato	0.23
Cucumber	0.18
Rice	0.02
Rice (cooked)	0.02

Table 10.1: Phosphatidic acid contents of several foodstuffs. Data taken from [437]. The original data was reported in μmol per g and has been converted into mg per g assuming a molar mass of 674.9 g/mol ($C_{37}H_{71}O_8P$).

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Box 10.1

The membranes of cells consist of a bilayer of lipids and proteins. Phospholipids are the most abundant lipids in membranes. Characteristic of phospholipids is that they have a polar (hydrophilic) head (the phosphate group) and two non-polar (hydrophobic) tails (usually fatty acids). In the case of glycerophospholipids, the fatty acids and the phosphate group are bound to a glycerol molecule.

The heads of phospholipids face the water, while the tails face each other. The phospholipid composition of a membrane is important to its function. A distinction is made between four different types of glycerophospholipids: phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. The differences mainly depend on what chemical group is bound to the phosphate group (the polar head). Phosphatidic acid functions as the 'simplest' glycerophospholipid: there's no chemical group attached to the phosphate group, just a hydrogen atom. Because of its structure, phosphatidic acid thus is an important precursor for other glycerophospholipids and is therefore a pivotal player in glycerophospholipid synthesis.

10.1.1 Biosynthesis

Phosphatidic acid holds a central role in membrame glycerophospholipid and triacylglycerol synthesis [153] and can be generated by three different metabolic pathways. One of

these pathways allows for the *de novo* synthesis of phosphatidic acid. This mechanism starts from two intermediate products of the glycolysis (see Section 2.3). During glycolysis, fructose-1,6-biphosphate is split into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The dihydroxyacetone phosphate can then be converted into glycerol-3-phosphate by the enzyme glycerol-3-phosphate dehydrogenase. Subsequently, two fatty acids can be bound to this glycerol-3-phosphate to form phosphatidic acid. The first acetylation reaction is catalyzed by glycerol-3-phosphate acyltransferase (GPAT). This yields lysophosphatidic acid (LPA) which can then be acetylated by the enzyme lysophosphatidic acid acyltransferase (LPAAT) to yield phosphatidic acid. The fatty acids used for this can also be derived from *de novo* fatty acid synthesis. However, this pathway appears to prefer the formation phosphatidic acid containing two saturated fatty acids [501]. This form does not appear capable of inducing mTORC1 activity [491].

In the second metabolic pathway, diacylglycerol is phosphorylated to phosphatidic acid by a diacylglycerol kinase or PRK-like ER kinase (PERK) [54], a kinase that is located on the membrane of the endoplasmic reticulum. Remarkably, the kinase activity of PERK is dependent on phosphatidylinositol 3-kinase (PI3K). PI3K is activated as a result of insulin or insulin-like growth factor 1 receptor activation, as described in Section 4.2.1. The diacylglycerol can be derived from triacylglycerol stored in adipose tissue. In that case, the acyl-CoA group is deacylated by a lipase. It can also be derived from phosphatidylinositol. In that case, phospholipase C cleaves the molecule at the phosphate group.

The third metabolic pathway that can yield phosphatidic acid uses phosphatidylcholine as a substrate. The enzyme phospholipase D hydrolyses phosphatidylcholine, which makes it lose its choline group.

The pathway that is important for mechanical stimuli-induced mTORC1 signaling appears to mainly rely on the diacylglycerol kinases, in particular the ζ-isoform [495].

10.1.2 Absorption

Phosphatidic acid is supplemented orally in the form of a capsule or a soft gel. It's metabolized to lysophospholipids or glycerol 3-phosphate in the small intestine. Phospholipases of the pancreatic juice are responsible for this. These phospholipases hydrolyze the ester bonds on the first (*sn*-1) or second carbon atom (*sn*-2) of phosphatidic acid. The phospholipases, in particular phospholipase A2, specifically cleave the fatty acid at the second carbon atom [454]. These products are then absorbed by the intestinal mucosa. In particular, this will comprise lysophospholipids lacking a fatty acid on the second carbon atom. In the enterocytes, the lysophospholipids can be re-esterified with a fatty acid which thus yields phosphatidic acid again. The fatty acid with which the lysophospholipid is esterified depends for a large part on the other fatty acids that are being absorbed by the enterocytes. It's therefore dependent on the supply of fat from the diet.

The phosphatidic acid that is now reformed in the enterocytes, and potentially other phospholipid products, are packaged into chylomicrons. The chylomicrons are then excreted by the enterocytes by means of exocytosis, after which it enters the lymphatic system. From the lymphatic system it eventually enters the blood circulation. However, because it travels via the lymphatic system, it enters the circulation more slowly than other nutrients and supplements that are transported to the liver via the portal vein and subsequently get into the circulation from the liver. In a single test subject a peak in the plasma phosphatidic acid concentration (T_{max}) was found after three hours, and this was still increased seven hours after ingestion [367]. This is in line with what one would

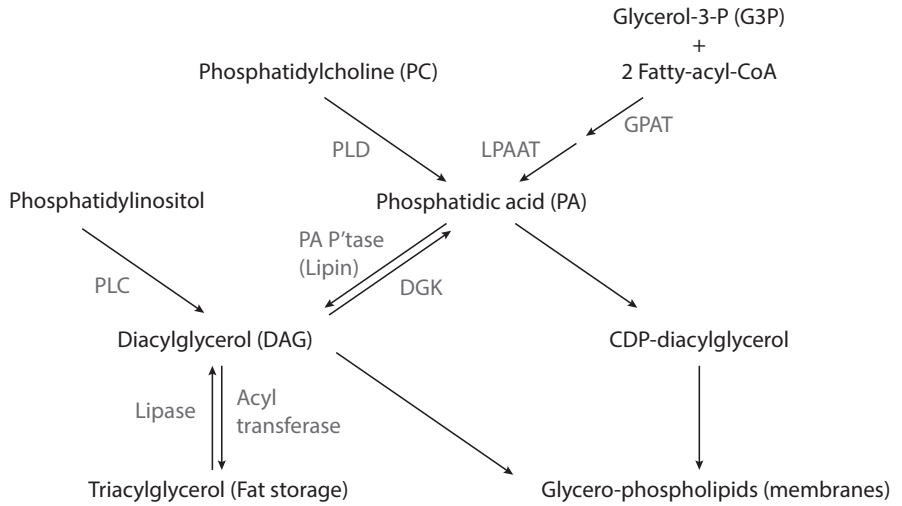


Figure 10.2: Phosphatidic acid (PA) can be synthesized from glycerol 3-phosphate (G3P), phosphatidylcholine (PC), and diacylglycerol (DAG). G3P is acetylated twice to form PA. Acetylation is first catalyzed by GPAT, followed by LPAAT. PC is hydrolyzed by PLD to yield PA, and DAG is phosphorylated by DGK to yield PA. DAG is derived from triacylglycerols and phosphatidylinositol. PA phosphatase is responsible for the dephosphorylation of PA, which converts it to DAG. Several CDP-diacylglycerol synthases convert PA into CDP-diacylglycerol. Figure taken from Bond [59].

expect with substances reaching the circulation via the lymphatic system. The maximum measured concentration (C_{max}) was 3.51 nmol/mL (the baseline concentration was 2.66 nmol/mL; thus a 32 % increase). The dose supplied in this study was 1.5 g soy-derived phosphatidic acid. A sharp increase in the plasma lysophosphatidic acid concentration was also seen.

However, at the time of writing, no data is available with regard to the incorporation of dietary phosphatidic acid into the membranes of muscle cells. Also note that there is evidence that phosphatidic acid supplementation leads, at least in part, to mTORC1 activation by extracellular conversion to lysophosphatidic acid [486]. This would imply that incorporation of phosphatidic acid into the cell membrane (or uptake by the cell) might not be necessary for its potential anabolic effects. Instead, the lysophosphatidic acid formed outside of the cell binds to endothelial differentiation gene 2 (EDG-2, a lysophosphatidic acid receptor) and this, in turn, stimulates mTORC1 signaling by activation of the ERK pathway.

10.1.3 Metabolism and excretion

Phosphatidic acid plays a central role in the synthesis of glycerophospholipids. It functions as the common precursor to glycerophospholipids. This important metabolic pathway that phosphatidic acid can follow begins with 'activating' it by attaching to cytidine diphosphate (CDP), yielding CDP-diacylglycerol. The activation with CDP is comparable to that of

glucose with uridine diphosphate (UDP) as seen in glycogen synthesis. Subsequently, the hydroxyl group can react with an alcohol, which can yield three different glycerophospholipids: phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. The amino acid serine can also react with the hydroxyl group to yield phosphatidylserine.

Additionally, phosphatidic acid can also be dephosphorylated by a phosphatidic acid phosphatase [84]. This yields a diacylglycerol molecule. This molecule can then be re-esterified with a fatty acid by acyltransferase. This creates a triacylglycerol that can be stored in, among other things, the adipose tissue. Diacylglycerol can also be transformed to glycerophospholipids.

In principle, the glycerol molecule and the attached fatty acids of phosphatidic acid can serve as substrates for energy production. However, the quantities are so small that they don't play a significant role in energy metabolism.

10.2 Mechanism of action

Section 4.2.4 has discussed how mechanical loading leads to the formation of phosphatidic acid. Phosphatidic acid can then stimulate mTORC1 activity through direct interaction with the complex. Dietary phosphatidic acid can also stimulate mTORC1 activity, but might do this in an additional manner as well compared to the phosphatidic acid formed by mechanical stress [413]. After all, the phosphatidic acid in response to mechanical stress is produced intracellularly, while the phosphatidic acid from the diet will also be located outside the cell.

10.2.1 Activation of mTORC1

In 2011, Yoon et al. conducted an *in vitro* experiment that would demonstrate that phosphatidic acid is able to directly activate mTORC1 [493]. First, it was determined that addition of phosphatidic acid vesicles to the cell medium led to mTORC1 activation. Next, the effect of phosphatidic acid on the endogenous mTORC1 inhibitor FKBP38 was determined. FKBP38, like phosphatidic acid, binds to the FRB domain. As such, phosphatidic acid might therefore bind competitively to this domain, making FKBP38 no longer able to bind it. Indeed, phosphatidic acid was found to compete with FKBP38 for binding to this domain, as a result of which it overrides the inhibitory effect that FKBP38 has on mTORC1. Finally, the researchers looked at whether phosphatidic acid was able to stimulate mTORC1 activity in the absence of FKBP38. If this were the case, it would not only override the inhibitory effect of FKBP38, but it would also activate mTORC1 itself. And, indeed, the researchers' results suggest that this is the case: phosphatidic acid also acts as an allosteric activator of mTORC1.

Later research, also by Yoon et al., demonstrated that phosphatidic acid leads to the dissociation of the mTORC1 inhibitor DEPTOR from the complex [491]. Additionally, they found that phosphatidic acid with atleast one unsaturated fatty chain attached to it was required for this, as the variants with two saturated fatty acids didn't. This is in line with the results of another *in vitro* experiment comparing the effect of soy-derived phosphatidic acid mTORC1 activation with that of egg-derived phosphatidic acid [246]. The soy-derived phosphatidic acid led to a stronger activation of mTORC1 and contained a higher amount of unsaturated fatty acids than egg-derived phosphatidic acid.

Furthermore, it has been suggested that exogeneous phosphatidic acid may not activate mTORC1 through direct interaction. Instead, extracellular conversion to lysophosphatidic

acid would need to take place first. Lysophosphatidic acid is formed by a phospholipase, which removes the acyl group attached to the second carbon atom. The formed lysophosphatidic acid can then bind to endothelial differentiation gene 2 (EDG-2) receptors that are located on the cell membrane [486]. EDG-2 is a receptor that belongs to the family of G-protein coupled receptors. Activation of the receptors activates the MEK-ERK pathway, which by inhibition of the TSC complex [290] and raptor [85], can stimulate mTORC1 signaling. In addition, EDG-2 activation stimulates the activity of phospholipase D, thereby leading to the generation of intracellular phosphatidic acid from phosphatidylcholine.

Later research tested the involvement of the MEK-ERK pathway by using the MEK-ERK inhibitor U0126 in an *ex vivo* model [494]. In this experimental model, U0216 inhibited the rise in S6K1 and 4E-BP1 phosphorylation that normally occurs in response to mechanical stimulation. However, this increase wasn't completely blocked. Similar results were found in C2C12 myoblasts that were exposed to phosphatidic acid. U0126 didn't block the increase in S6K1 phosphorylation, nor in 4E-BP1 phosphorylation, but did inhibit it somewhat. In conclusion, the MEK-ERK pathway isn't necessary for the mechanical stimuli-induced increase in mTORC1 activity, but it does appear to be partly responsible for it.

10.2.2 Inhibition of ubiquitin ligases

An important mediator of muscle atrophy is the ubiquitin system. This system consists of an interplay of molecules that tag proteins with the marker ubiquitin and a large protein complex that subsequently breaks down these tagged proteins, the proteasome. Two so-called E3 ligases, which are directly responsible for binding ubiquitin to proteins, appear to primarily be involved in this regulation in muscle cells. These are muscle atrophy F-box (MAFbx, also known as atrogin-1), and muscle ring finger 1 (MuRF1). An increased expression of these two ubiquitin ligases is found in many experimental models of muscle atrophy, such as cachexia or dexamethasone (a corticosteroid) administration. For this reason, they are often used in experimental research as a surrogate marker of muscle breakdown. The forkhead box class O (FoxO) family of transcription factors plays an important role in the regulation of their expression.

Overexpression of phospholipase D, which generates phosphatidic acid from phosphatidylcholine, leads to decreased mRNA expression of FoxO, MAFbx, and MuRF1 in fully differentiated L6 myotubes [236]. Incubation of myotubes with 100 % μM phosphatidic acid also inhibits the dexamethasone-induced increase in MuRF1, MAFbx, and FoxO3 mRNA. In addition, the same study shows that exogenous phosphatidic acid was capable of counteracting the atrophy caused by dexamethasone and TNFα (a pro-inflammatory cytokine). A possible mechanism that could underlie this effect might be mediated via mTORC2. The role of mTORC2 in the regulation of muscle growth is less prominent, and structurally the complex differs from mTORC1 in that it's associated with the protein rictor instead of raptor. Because of the direct interaction of phosphatidic acid with mTOR, it might also affect the activity of mTORC2. mTORC2 phosphorylates, and thereby activates, Akt on a serine residue [401]. Once Akt is activated, it phosphorylates several substrates, including FoxO proteins [444]. MuRF1 and MAFbx are two important downstream effectors of FoxO signaling. But phosphorylation of FoxO proteins by Akt inhibits their function, and thus that of its downstream effectors. If this pathway via mTORC2 and Akt lies at the root of phosphatidic acid's muscle atrophy inhibiting effect in these models, the question remains how relevant this is under physiological conditions. After all, under

physiological conditions growth factors that activate the PI3K-Akt pathway will be present, and it's not necessarily to be expected that this effect is additive.

10.3 Clinical results

In 2012, the first clinical trial was conducted that looked at the effects of phosphatidic acid on strength, muscle thickness and lean body mass [217]. In total, sixteen men with resistance exercise experience participated in the trial. Seven of these men received phosphatidic acid (750 mg daily). The remaining nine men were given a placebo. At the start of the trial, body composition was determined with dual-energy X-ray absorptiometry (DXA). The 1-RM of the squat and bench press were determined as measures of strength. The muscle thickness and architecture (pennation angle) of the vastus lateralis were determined using ultrasonography. These measurements were repeated after the 8-week resistance exercise program, which comprised of four workouts a week. The results of this are summarized in Table 10.2.

None of the seven outcome measures reached statistical significance, but a trend ($P = 0.065$) was noted for an increase in lean body mass in the phosphatidic acid group compared to placebo. When using the controversial statistical method magnitude-based inference, a 'very likely' positive effect on lean body mass and a 'likely' positive effect on the 1-RM squat was found. For the other five outcome measures the data was too unclear to infer anything (see also Box 10.2). The limited group size and the results of the MBI might suggest that the small sample sizes obscured a potentially true effect on lean body mass. Noteworthy is that the placebo group experienced practically no change in lean body mass (+0.1 kg), which might indicate that the training stimulus wasn't adequate enough to invoke muscle hypertrophy.

Outcome measure	Δ	P value	Clinical interpretation
1-RM bench press (kg)	2.38	0.43	Unclear
1-RM squat (kg)	4.31	0.19	Likely
Muscle thickness (cm)	0.007	0.96	Unclear
Pennation angle (°)	0.79	0.69	Unclear
Body mass (kg)	1.4	0.35	Unclear
Body fat(kg)	0.1	0.99	Unclear
Lean body mass (kg)	1.6	0.065	Very likely

Table 10.2: Mean differences (Δ, phosphatidic acid vs placebo) and P values for group-×-time interactions. Analysis was performed using a two-way mixed factorial analysis of variance (ANOVA) with Tukey post-hoc tests. The α value is set at 0.05. Clinical interpretation based on magnitude-based inference (see Box 10.2). Results from [217].

Box 10.2

Magnitude-based inference is a statistical method that is increasingly being used in exercise science in recent years. This method is mainly used to make statements about small effects in small groups that are otherwise unable to achieve statistical significance due to a lack of statistical power. The method is a lot less conservative than traditional methods. In this way, it's postulated

that it can form a good solution for the high probability of type II errors ('false negatives') with small effects in small sample sizes. The generated P values generated by this method are translated into descriptive categories, or interpretations, namely 'most unlikely', 'very unlikely', 'unlikely', 'possibly', 'likely', 'very likely', and 'most likely'. If the confidence interval (CI, commonly the 95 % CI) overrides the threshold of both a positive and negative effect, it's classified as 'unclear'.

However, the statistical method is not without its objections and its use is discouraged [476]. Caution is therefore advised when interpreting the results of MBI. It must be acknowledged that small-scale studies simply have limited statistical power.

Following this first clinical trial, a similar study was conducted by Joy et al. [246]. Again, an 8-week resistance exercise program was followed by trained men and 750 mg of phosphatidic acid per day was compared to a placebo. Some differences were that the number of participants was a bit larger (a total of 28), phosphatidic acid intake was timed, and a direct measure of muscle hypertrophy was performed (muscle cross-sectional area [CSA]). Phosphatidic acid was taken thirty minutes pre-workout (450 mg) and immediately post-workout (300 mg) on training days. On rest days, 450 mg was taken with breakfast and 300 mg with dinner. Lean body mass, determined by DXA, showed a significant group-$\times$-time interaction in which the phosphatidic acid group showed a larger increase compared to the placebo group. In contrast to the previous study, lean body mass in the placebo group now also increased (+1.2 kg). This suggests the training stimulus was adequate. A trend was found for the change in fat mass, which decreased more in the phosphatidic acid group. A significantly greater increase in CSA, determined by ultrasound of the rectus femoris, was also found in the phosphatidic acid group. Similar to the previous study, an 1-RM bench press was used as a measure of upper body strength. However, instead of an 1-RM squat, an 1-RM leg press was used as a measure of lower body strength. The 1-RM leg press increased significantly more in the phosphatidic acid group compared to the placebo, but this wasn't observed for the 1-RM bench press. The results of this study are in line with the previously conducted pilot study.

A third study was conducted by Escalante et al. [143]. A total of eighteen trained men participated in the study. Just as the previous two trials, an 8-week resistance exercise program was followed and DXA was used to measure changes in body composition. DXA was also used to regionally assess thigh muscle mass. The dosage and timing of intake was the same as that in the trial by Joy et al. However, an important difference is that the supplement used in this study also contained vitamin D, leucine and the leucine metabolite HMB. The study found a significant increase in the 1-RM bench press, 1-RM leg press, and lean body mass, and a trend towards a decrease in fat mass, in the phosphatidic acid group compared to the placebo group. No significant difference was found in thigh muscle mass hypertrophy between both groups. While these results are consistent with previous research, they are confounded by the addition of vitamin D, leucine, and HMB. It's therefore not possible to say how much phosphatidic acid contributed to these results.

A more recent clinical trial looked at the effects of lower dosages of phosphatidic acid (250 and 350 mg daily) in combination with resistance exercise [14]. A total of 28 subjects participated in the trial, of which 10 received a placebo, 9 received 250 mg phosphatidic acid, and 9 received 375 mg phosphatidic acid. Again, all subjects were trained and an 8-week resistance exercise program was followed. The supplement was taken one hour pre-workout on training days and in the morning on rest days. DXA was used to determine

fat mass and lean body mass. Ultrasonography was used to determine the CSA of the rectus femoris. Lower body strength was assessed by a 1-RM leg press, while upper body strength was not included as an outcome measure in this study. An ANOVA didn't show a significant group-$\times$-time interaction for any of the outcome measures. The authors thus used MBI, which showed a likely positive effect on lean body mass and CSA of the rectus femoris, and a very likely positive effect on the 1-RM leg press in the phosphatidic acid group receiving 250 mg daily compared to the placebo group. The 375 mg group also showed a likely positive effect on CSA of the rectus femoris, and 1-RM leg press, and a possible positive effect on lean body mass compared to the placebo group. However, the reported MBI results don't appear to be accurate. The lean body mass increased, on average, less in both phosphatidic acid groups (+0.5 and +1.3 kg) than in the placebo group. Fat mass also decreased more on average in the placebo group. The other numbers in their table reporting the MBI values also don't appear to agree with the numbers reported elsewhere in the paper.

Given the ambiguous results in the literature, Gonzalez et al. also conducted a trial to assess the effect of phosphatidic acid supplementation (750 mg daily) in trained men [173]. A total of fifteen trained men participated in the study and here they also followed a (supervised) resistance exercise program. Half the PA dose was taken 30 min pre-workout and the other half was taken immediately post-workout. Muscle thickness of the rectus femoris, vastus lateralis, biceps brachii and triceps brachii muscles were measured via ultrasonography and 1-RM of squat, deadlift and bench press were performed as strength measures. Although all participants made improvements in each measure of muscle thickness and strength, no significant differences between the PA and placebo group were found. Unfortunately, body composition wasn't determined.

An overview of the results of these trials is presented in Table 10.3.

Study	**1-RM BP (kg)**	**1-RM SQ/LP (kg)**	**LBM (kg)**	**FM (kg)**
Gonzalez et al. (2017)	$+0.5$	$+0.6$	-	-
Andre et al. (2016)[a]	-	$+20.5$	-0.3	$+0.4$
Andre et al. (2016)[b]	-	$+42.4$	-1.1	$+0.3$
Escalante et al. (2016)[c]	$+8.5^*$	$+29.2^*$	$+1.1^*$	-1.0
Joy et al. (2014)	$+10.3$	$+19.5^*$	$+1.2^*$	-0.8
Hoffman et al. (2012)	$+2.4$	$+4.2$	$+1.6$	$+0.1$

Table 10.3: Results presented as the mean difference of change between the phosphatidic acid group and placebo group after an 8-week resistance exercise program. The used dosage was 750 mg daily, unless stated otherwise. [a] Used dosage was 375 mg daily. [b] Used dosage was 250 mg daily. [c] In addition to 750 mg phosphatidic acid, subjects also received vitamin D, leucine, and HMB. * Significant difference compared to placebo group ($P < 0.05$). Abbreviations: BP, bench press; SQ, squat; LP, leg press; LBM, lean body mass; FM, fat mass.

In summary, the studies suggest that phosphatidic acid might have a positive effect on lean body mass and muscle strength when combined with resistance exercise. The recent study by Andre et al. is plagued by incorrectly reported data and the lower dose may also play a role in their results. This could've prevented the trial from finding an effect. Both Escalante et al. and Joy et al. found a positive effect on lean body mass by phosphatidic acid, although the trial by Escalante et al. combined phosphatidic acid with vitamin D,

leucine and HMB. This could have affected the results, making it impossible to say to what extent phosphatidic acid was responsible for the results. The results of Hoffman et al. only demonstrate a positive effect when the controversial statistical method MBI is used. Finally, most studies demonstrate a positive effect on muscle strength, although this only reached significance in the trial by Escalante et al. (in which it was combined with other supplements). The recently published trial by Gonzalez et al. found no effect on strength nor muscle thickness.

Future research with more test subjects is needed to provide more certainty. If there is an effect on lean body mass or strength, it's probably small. To date, no research has evaluated the effects of phosphatidic acid in an untrained population. Thus, it remains undetermined what the effect of phosphatidic acid is in untrained individuals. It's also unclear wether the the mechanism of action, stimulation of mTORC1 through interaction with mTOR and activation of the MEK-ERK pathway, plays a role in humans *in vivo*. Given the inhibitory effect of phosphatidic acid on ubiquitin ligases in fully differentiated L6 myotubes, it's interesting to investigate the effect of this supplement in populations which are affected by muscle-wasting conditions.

10.4 Safety

Remarkably little is known about the safety of phosphatidic acid. To date, only one publication in the scientific literature has directly addressed its safety [133]. It was presented as a poster presentation at the tenth annual conference of the International Society of Sports Nutrition in 2013. Phosphatidic's safety was evaluated in 28 healthy young men. The study was double-blind, with one group receiving 750 mg of soy-derived phosphatidic acid daily, and the other group receiving a placebo. Measures of cardiovascular, kidney, and liver function were analyzed with a full comprehensive metabolic panel (CMP) and a complete blood count (CBC). A urine sample was also taken to determine specific gravity (a kidney function test) and its pH. After eight weeks of supplementation, no significant difference was found between the phosphatidic acid and the placebo group.

None of the clinical trials covered in Section 10.3 reported an adverse event. However, these trials didn't examine phosphatidic acid's safety specifically, and as such no additional testing was done to detect certain changes that might be relevant (for example, a potential increase in LDL cholesterol).

With the limited data there is, phosphpatidic acid supplementation appears to be safe at a dosage of 750 mg daily in the short-term. Further research is necessary to gain more certainty about the safety of the supplement, including different study populations as well as its long-term safety.

10.5 Conclusion

Phosphatidic acid might be an interesting supplement for those who wish to increase their muscle mass or gain more strength. A plethora of molecular research implies a close involvement of phosphatidic acid in the regulation of the mTORC1 protein complex and thus also in protein synthesis. Since it's present in the diet in limited quantities, supplementation would be necessary to arrive at a dosage that is proposed to have an ergogenic effect. The usual dosage is 750 mg daily of soy-derived phosphatidic acid. In the absence of comparative studies on optimal timing, it's advisable to adhere to the timing of

supplementation that's frequently used in the clinical trials. On training days, phosphatidic acid is taken thirty to sixty minutes pre-workout and immediately post-workout. The dosage is split into 450 mg pre-workout and 300 mg post-workout (thus totaling 750 mg). On rest days, 450 mg is taken with breakfast and 300 mg with dinner. Because phosphatidic acid is processed after ingestion in the gastrointestinal tract, and subsequently re-esterified in the enterocytes to phosphatidic acid, the composition of the meal with which it's ingested likely affects its effectiveness. However, no research has looked into this. Based on the fact that phosphatidic acid esterified with unsaturated fatty acids is particularly effective in stimulating mTORC1 activity, it may be useful to avoid simultaneous intake with saturated fatty acids.

11. Trimethylglycine

11.1 Introduction

Trimethylglycine (TMG) is a methyl derivative of the amino acid glycine. TMG was first discovered in the juice of the sugar beet (*Beta vulgaris*), to which it owes its alternative name betaine. TMG can also be found in animals, plants and microorganisms. Especially crustaceans—marine invertebrates in particular—, wheat germ or bran, and spinach are rich sources of TMG [105].

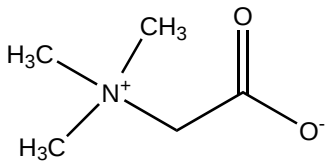


Figure 11.1: Structural formula of trimethylglycine. The molecule is zwitterion, as it has both a negative charge (the carboxyl group) and a positive charge (the quaternary ammonium cation).

The supplement gained some popularity after a 2013 study showed that it significantly improved body composition, arm size, and bench press capacity in resistance-trained men [95].

The supplement's action is attributed to two mechanisms. For example, TMG can act as an osmolyte in the cell. Osmolytes are tightly involved in the regulation of the osmotic pressure in a cell, and thus their fluid balance. This effect is the result of osmosis, in which water diffuses from outside the cell to inside the cell (where the concentration of the osmolyte is higher). Osmosis is further explained in Box 11.1. TMG accmulates in almost all tissues to regulate the cell volume and is viewed as one of the most important organic osmolytes [272]. TMG thus maintains the fluid balance of cells.

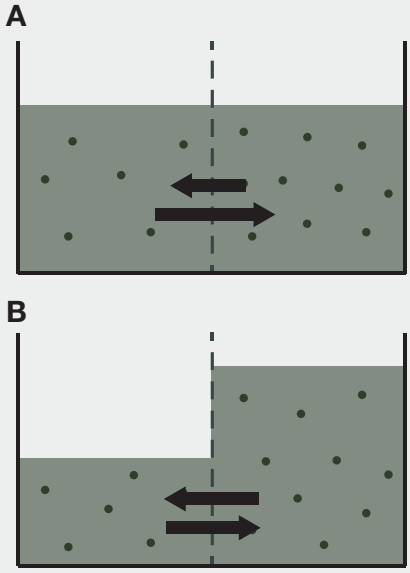
Additionally, TMG acts as a methyl donor: it feeds a biochemical process called transmethylation. A methyl group of TMG is passed onto another molecule. Methylation

reactions are important for numerous cellular processes and it's therefore important that sufficient methyl groups are available to feed these reactions. In particular, TMG acts as a methyl donor for the remethylation of homocysteine, which yields methionine. TMG supplementation is therefore effective in lowering the serum homocysteine concentration [308]. A high serum homocysteine concentration is considered an independent risk factor of cardiovascular disease. However, the direct effect of TMG supplementation on cardiovascular disease risk is currently unknown [369]. A recent meta-analysis also shows an association between the level of TMG intake and cancer incidence [430]. A higher intake would confer a protective effect.

Box 11.1

i

Osmosis is a phenomenon that's central to the action of osmolytes, such as TMG. Basically, it's a form of passive transport, as described in Section 3.2.1. However, it aren't the molecules dissolved in water that diffuse across the membrane, rather, it are the water molecules themselves. When a semipermeable membrane that allows water to pass through it is present, but not a (selection of) the particles dissolved in it, osmosis can occur if there's a different in concentration between both sides of the membrane of these particles. This is visualized in the figure below.



As a result of the thermal movement, particles, both the impermeable as well as the water particles, continuously come into contact with the membrane. If there are more impermeable particles on one side than the other side, less water particles come into contact with the membrane compared to the other side. This results in more water particles moving from one side to the other. So, water moves from a dilute solution (low concentration of a solute) to a concentrated solution (high concentration of a solute).

In the figure, A has a higher concentration of solute on the right side than on the left. So there will be a net movement of water from the left to the right. However, because water will move to the other side of the membrane, an osmotic pressure difference also arises because more fluid 'presses' on the membrane (not illustrated in the figure). Because water flows from a low

concentration solution to a higher concentration solution, the concentration difference will become smaller and smaller and the increased fluid volume on the other side will also increase the osmotic pressure on that side. Eventually an equilibrium will be reached, so that no net water flow will take place across the membrane anymore (as shown at B in the figure). The osmotic pressure can be calculated using the Van 't Hoff equation, but will not be discussed further here.
By actively regulating the concentration of an osmolyte in the cell, a cell can make smart use of osmosis. After all, water 'follows' the highest concentration of the osmolyte. So when a cell accumulates an osmolyte inside of it, it will also 'suck' water into it (and vice versa).

Product	Amount of TMG (in mg/100 g)
Wheat bran	1506
Spinach, cooked	725
Spinach, raw	675
Beets, canned	334
Shrimp, canned	246
White bread	105
Sweet potato	35
Beer	10
Roasted chicken	6
Rice	<1

Table 11.1: Amount of TMG present in several foodstuffs. Data taken from [498].

11.1.1 Biosynthesis

TMG is produced in the human body by the liver and kidneys. Choline is required for this, which can be obtained from the diet or formed from the amino acid serine. Choline can only be found in small quantities in food. The largest contribution of dietary choline therefore stems from the phospholipid phosphatidylcholine (lecithin), where choline functions as the head of the phospholipid. TMG can be formed from choline by two successive oxidation reactions. First, choline is oxidized by the enzyme choline dehydrogenase to form betaine aldehyde. After this, the betaine aldehyde is oxidized to TMG by the enzyme betaine-aldehyde dehydrogenase. Both reactions take place in the mitochondria. The flux-regulating step of the process is probably the transport from choline into the mitochondria by the choline transporter [336].

11.1.2 Absorption

TMG is ingested orally, often in the form of capsules, but sometimes also as a powder dissolved in water. In addition, it's present in various dietary sources, in particular fish and crustaceans—marine invertebrates in particular—, wheat germ or bran, and spinach [105]. A regular daily TMG intake is estimated to be 100–300 mg [278]. After ingestion, TMG is rapidly absorbed with a peak concentration in the blood approximately 2 hours after ingestion [410, 25]. TMG from food is absorbed slightly more slowly than when it's supplemented directly, but the bioavailability of TMG in supplement form and from food is the same [25]. TMG is transported by Na^+- and Cl^--dependent secondary transport

(described in Section 3.2.2), as well as Na^+-independent passive transport (described in Section 3.2.1) [105, 254]. The betaine-γ-aminobutyric acid transporter and the amino acid transport system A are likely responsible for its transport, although little is known at the time of writing about the transport across the sarcolemma.

Kidney and liver cells accumulate TMG in response to hyperosmotic shrinking of a cell, and release TMG upon hypo-osmotic cell swelling [258]. The role of TMG as an organic osmolyte is obvious under these conditions. The tightly regulated transport of TMG across the cell membrane provides the cell with a means of responding to changes in cell volume. This is especially important for kidney cells, which, due to their filtering function of the blood, are exposed to high osmotic pressure. However, it should be noted that the role of TMG as an osmolyte in the liver, in healthy individuals, is of much less importance. The liver experiences little variability in its osmotic pressure, making it unlikely that TMG *in vivo* is actively being regulated by the liver cells to accommodate osmotic pressure changes. In the liver, the role of TMG as a methyl donor seems particularly important. A role that's in line with the metabolic function of the liver.

The TMG plasma concentration demonstrates sex differences and strong interindividual variability. In women, the concentration is about 20–60 μmol/L and in men 25–75 μmol/L [278]. A small-scale trial in 8 men showed a 52 μmol/L increase in the TMG concentration after ingestion of 500 mg TMG [25]. When receiving 517 mg TMG from food, a maximum increase of 39μmol/L was measured. The TMG serum concentration also shows a dose-dependent effect that is linear at doses of 1, 3, and 6 g [409].

11.1.3 Metabolism and excretion

TMG has a high bioavailability. Only a small portion is ultimately cleared by the kidneys and recovered in the urine [410]. The supplementation of TMG leads to a slight short-term increase in TMG in the urine, but after two weeks of supplementation, this increase disappears [278].

TMG can also be lost through sweating. In a small-scale study, TMG concentrations in sweat were found to be approximately seven times higher than in plasma (232 $\pm$84 μmol/L vs 34 $\pm$11 μmol/L) [106]. Given a molar mass of 117 g/mol, this equates to a loss of 27 mg TMG per liter of sweat. Under normal circumstances, this loss will form a small part of total TMG intake. However, in athletes, and certainly under warm conditions, substantial amounts of TMG can be lost via the sweat. For example, sweat losses of eight to ten liters per day have been reported in professional and university American football players [168]. Nevertheless, with most recreational forms of sport, significantly less fluid is lost by sweating.

However, the majority of TMG is metabolized. TMG is converted into dimethylglycine (DMG) by the enzyme betaine-homocysteine methyltransferase (BHMT). In this process, a methyl group of TMG is transferred onto homocysteine, which thus yields methionine. The enzyme is found in the liver and kidneys [431]. The two products of the BHMT-catalyzed reaction (methionine [12] and DMG [151]) inhibit the activity of the enzyme; this is a form of feedback inhibition. DMG is the strongest inhibitor of BHMT activity of the two.

Subsequently, DMG is metabolized in the mitochondria by dimethylglycine dehydrogenase (DMGDH). This yields sarcosine, which is then converted into glycine by sarcosine dehydrogenase (SDH). In both reactions, tetrahydrofolate acts as a carbon acceptor, forming 5,10-methylenetetrahydrofolate. The latter carries a formaldehyde group that can be used for pyrimidine synthesis (cytosine, thymine and uracil, the building blocks of DNA

and RNA).

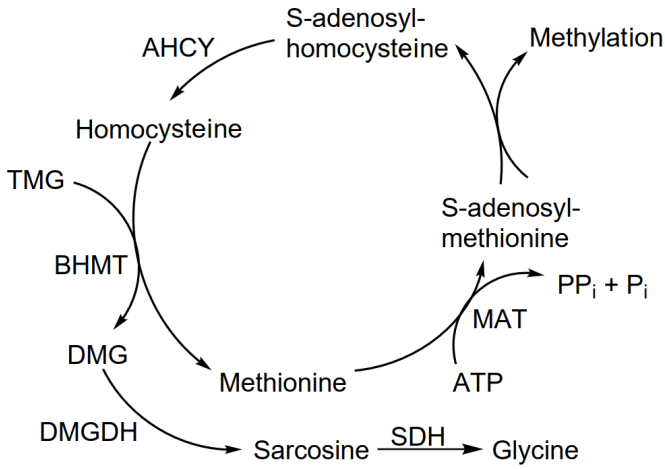


Figure 11.2: The methionine cycle. A methyl group of TMG is transferred onto homocysteine, yielding methionine and DMG. This reaction is catalyzed by the enzyme BHMT. DMG can be further metabolized to sarcosine by SDH. This reactions yields glycine. The formed methionine can be activated by forming SAM. This reaction consumes an ATP molecule and is catalyzed by MAT. It cleaves of the three phosphate groups of the ATP molecule. The formed SAM can then be used as a methyl donor for a variety of cellular reactions. When SAM donates a methyl group, S-adenosylhomocysteine is formed. This molecule is then cleaved into homocysteine and adenosine (not shown) by the enzyme AHCY. Abbreviations: TMG, trimethylglycine; DMG, dimethylglycine; BHMT, betaine-homocysteine methyltransferase; DMGDH, dimethylglycine dehydrogenase; SDH, sarcosine dehydrogenase; MAT, methionine adenosyltransferase; ATP, adenosine triphosphate; PP_i, pyrophosphate group; P_i, phosphate group; AHCY, adenosylhomocysteinase.

11.2 Mechanism of action

It's assumed that the positive effect of TMG on body composition is, at least in part, due to its role as an osmolyte and methyl donor. On the one hand, the accumulation of TMG in the muscle cells would lead to the cells swelling as a result of osmosis. This swelling of the muscle cells is said to function as an anabolic signal that stimulates protein synthesis. On the other hand, as a methyl donor, TMG would have a positive effect on biochemical processes that would ultimately stimulate muscle growth. In addition, some chemical properties that TMG possesses, as a result of its trimethyl nitrogen group, would play a role.

However, it should be emphasized that the quantitative contribution of each of these mechanisms to its positive effects on body composition is unknown. It's as yet largely unclear which mechanisms actually play a role *in vivo*.

11.2.1 Promotion of glycolysis

Glycolysis (see Section 2.3), the biochemical process by which glucose is split into two molecules of pyruvate and thus generates ATP, plays an important role in both relatively

short intense efforts and endurance sports. For glycolysis to take place, the electron carrier NAD^+ is required as an oxidant (electron acceptor), in which case it's reduced to NADH. In addition to the availability of the other substrates for glycolysis, namely glucose, ADP and P_i, the ratio NAD^+:NADH also determines the rate with which this process takes place (see Reaction 2.3).

It's hypothesized that molecules that contain an electrophilic methyl group (EMG) attract the nucleophilic hydrogen ion (H^-) of NADH, again generating NAD^+ [162]. Since TMG has an EMG, it could therefore also attract the nucleophilic hydrogen ion of NADH. This produces NADH, DMG, and methane [94].

The pyruvate formed by glycolysis can be further oxidized in the mitochondria, or be reduced to lactate or transaminated to alanine in the cytosol (see Section 2.3.1).

A clinical study evaluated the effect of TMG supplementation on running performance in trained distance runners [20]. Subjects were dehydrated (-2.7 % of total body weight) and then given 1 liter of fluid (6 % carbohydrates) to rehydrate (to -1.4 % of total body weight). Subjects were given this fluid with or without 5 g of TMG before running on the treadmill for 75 minutes (65 % VO_2max), followed by a sprint to exhaustion (84 % VO_2 max). The plasma lactate concentration was determined during the running on the treadmill as well as immediately after sprinting. There was no difference in the plasma lactate concentration between the two groups while running on the treadmill. However, immediately after the sprint, the plasma lactate concentration was significantly higher in the group receiving TMG. This might indicate an increase in the rate of glycolysis, an increased clearance of lactate into the circulation by the muscle cells, or a decreased clearance by organs such as the liver.

Another study, in which twelve men were subjected to a high-intensity training program, including exercises such as the bench press, squat jumps, and knee bends, didn't find an increased plasma lactate concentration in the subjects receiving TMG [276]. This contrast with the other study might be related to differences in intensity and duration of the training program, as well as the hydration status of the subjects. If the latter is the reason for the difference, it would mean that the role of TMG as an osmolyte is central to the effect on the plasma lactate concentration after exercise and thus possibly glycolysis.

However, yet another study showed a slightly smaller increase in the plasma lactate concentration in the TMG group compared to the placebo group [446]. In this study, the plasma lactate concentration was determined before and after performing ten sets of bench presses to failure on a machine. A slightly greater absolute decrease in muscle oxygen saturation was also seen in the TMG group. Taken together, this suggests that there is a shift in energy generation to the oxidative route. It's unclear what causes the differences between the studies.

11.2.2 Creatine synthesis

Creatine is synthesized in the human body from the two amino acids glycine and arginine (see Section 8.1.1). This synthesis takes place in two steps, with the final step being the transfer of a methyl group from S-adenosylmethionine (SAM) onto guanidinoacetic acid—which yields creatine and S-adenosylhomocysteine. SAM thus functions as the methyl donor in this reaction and methionine is activated to SAM with the help of ATP. Because TMG acts as a methyl donor for the remethylation of homocysteine to methionine, it has been hypothesized that it may stimulate creatine synthesis. Research in broilers showed an increase in liver creatine levels when fed a diet with added TMG [500]. However, a

clinical trial could not detect an increase in muscle creatine phosphate levels after ten days of TMG supplementation (2 g daily) [118].

11.2.3 Protein stabilizer

Proteins consist of a series of amino acids linked together by peptide bonds. These proteins fold into a certain spatial structure (conformation) in order to be able to perform a specific biological function. Enzymes, for example, adopt a conformation that ensures that a chemical reaction proceeds at a much faster rate (catalysis). Such a conformation is the result of the sum of many weak interactions between the amino acids and the environment, such as the cytosol in the cell that mainly consists of water. However, there are factors that can change the confomration of a protein, causing the protein to lose its biological function. This process is called denaturation—the protein unfolds into a denatured form. Denaturation of a protein can occur by a rise in temperature (heat shock), but also by chemical denaturants such as sodium dodecyl sulphate, which is used to pre-treat proteins for electrophoresis (see Box 1.4), and urea, a product of amino acid metabolism. Chemical denaturants generally compete with water to form hydrogen bonds with polar grouops on the proteins in order to denature them.

TMG has been found to protect the enzyme citrate synthase against denaturation by a rise in temperature *in vitro* [78]. It also promotes the renaturation (the 'folding back' to the correct conformation after denaturation) of citrate synthase after denaturation by urea. This protein stabilizing function of TMG could potentially maintain the flux of the citric acid cycle at a higher rate (see Section 2.4.1) under conditions in which a rise in temperature occurs or where the muscle cells are exposed to an increased urea concentration, such as is the case with intense exercise. This could promote sports performance under these conditions. However, clinical research supporting this line of thinking is lacking. Nevertheless, an increased VO_2 was measured during a sprint till exhaustion after 75 minutes of running on a treadmill in the subjects of the previously mentioned study in Section 11.2.1 [20]. Urea also hinders the ATPase activity of the myosin heads, an effect that is also counteracted by TMG [340].

11.2.4 Modulation of gene expression and phosphorylation state

Research suggests that TMG can affect the gene expression of some genes involved in muscle growth. In a small-scale clinical trial, 12 subjects received 2.5 g TMG or a placebo [17]. The trial was set up as a crossover design, with a washout period of 2 weeks. The intervention/supplementation period also lasted 2 weeks. A muscle biopsy was taken both before and after the supplementation period, and an extra muscle biopsy was taken post-workout after the supplementation period. Total Akt, Akt-P (Ser473), total S6K1, S6K1-P (Thr389), and AMPK-P (Thr172) were determined.

Total Akt increased significantly in the TMG group compared to the placebo group at rest. No difference was found in Akt-P and S6K1 at rest, but there was a decrease in Akt-P and S6K1-P post-workout in the placebo group that didn't occur in the TMG group. No difference in AMPK-P was found between the placebo group and TMG group.

In addition to looking at these markers of the mTORC1 pathway, blood was drawn to investigate the effect of TMG on, among other things, growth hormone (GH), insulin-like growth factor 1 (IGF-1), insulin and cortisol. TMG was found to have no effect on insulin, but resulted in a significant increase in IGF-1, a trend towards an increase in GH ($P = 0.067$), and a significant decrease in cortisol. An *in vitro* study in C2C12

myoblasts (mouse myoblasts) also showed an increase in IGF-1 receptor (IGF-1R) protein after incubation with TMG [412]. An increase in Akt and myosin heavy chain, the protein that forms the thick filament of the sarcomeres (see Section 1.2) was also observed. The increase of IGF-1 in the circulation after TMG supplementation, as well as the increase in IGF-1R that occurs after incubation of C2C12 myoblasts with TMG, make the IGF-1 pathway a potential candidate for the effect on the phophosorylation state of Akt (see Section 4.2.1).

11.3 Clinical results

Several clinical trials have looked at the effect of TMG supplementation on outcome measures such as strength, power, training volume and body composition. The majority of these studies, however, lasted only for a short period of time. Ranging from ten to fifteen days [216, 276, 215, 446, 118]. The supplementation period in the recently published study by Cholewa et al., however, was a total of six weeks [95].

A total of 24 young men participated in a double-blind, placebo-controlled trial by Hoffman et al. [216]. The young men were recreationally active and had a minimum of 3 months of experience with resistance exercise. The TMG group received 2.5 g daily, and the supplementation period lasted 15 days. Besides the baseline measurement, measurements were taken on days 7 and 8 (T2), and days 14 and 15 (T3). The researchers were particularly interested in the effect of TMG on muscle power and endurance. To assess upper body muscle power, bench press throw was used. The vertical jump was used to assess the lower body. No significant difference was found between the groups for muscle power of either the lower body or upper body. Endurance was determined by assessing the number of bench press and squat repetitions (both at 75 % 1-RM) until failure. No differences were found between the groups with regard to the bench press. However, the number of repetitions on the squat was significantly higher in the TMG group compared to the placebo group at T2. When looking at the total number of repetitions, a trend ($P = 0.06$) was detectable in favor of the TMG group at T3. Finally, no effect on muscle soreness was detected. Taken together, these study results suggest a positive effect on muscle endurance. A later small-scale study by the same research group tested the effect of TMG on isokinetic strength of the upper body [215]. No effect was found here.

Very comparable to the study of Hoffman et al., was a study by Lee et al [276]. In this study, men with some resistance exercise experience also received TMG (2.5 g daily) for 2 weeks. The isometric bench press force and bench press throwing power were significantly higher in the TMG group compared to the placebo group after the intervention. The isometric squat force was higher after the intervention versus before in the TMG group, but not in the placebo group. The TMG group also improved their vertical jujmp in two of the four sets. Some other outcome measures, such as the squat jump and squat and bench press repetitions to failure, showed no differences. This seems in conflict with the results of Hoffman et al, since they did show an improvement in lower body muscular endurance. However, they squatted at difference intensities: 75 % 1-RM in the study by Hoffman et al. and 85 % 1-RM in the study by Lee et al. In addition, the number of subjects in this study was smaller (only 12). That makes a type II error ('false negative') a possibility.

Trepanowski et al. conducted a double-blind cross-over trial with 13 resistance-trained men [446]. The subjects in this study also received 2.5 g of TMG daily for 2 weeks. The washout period lasted 3 weeks. As in previous research, muscle power of the lower

body (vertical jump) and upper body (bench press throw) was assessed. The maximum isometric strength of the lower body (leg press) and upper body (bench press) was also tested. Muscle endurance was determined by performing ten sets of lying machine bench press. After these ten sets were completed, oxygen saturation of the shoulder muscle was measured, since it was thought that TMG might improve blood flow to the muscles. As such, this could be reflected in an improved oxygen saturation of the muscle after exercise. Nevertheless, nothing statistically significant was found with a repeated measures analysis of variance. However, a significant improvement was found in total bench press repetitions as a result of supplementation (paired T-test). This wasn't found in the placebo group and is suggestive of improved muscle endurance. The oxygen saturation was slightly higher after TMG supplementation at the start of the muscle endurance test. This also led to a greater difference in oxygen saturation when compared to after the muscle endurance test (Δ oxygen saturation). As with previous research, the small sample size might have played a role in the lack of statistically significant results with the analysis of variance.

The most interesting clinical trial to date comes from Cholewa et al. [95]. This study differed from the previous studies in two important ways. One being that the supplementation period was six weeks this time, instead of only two. Another being that the participants were men with extensive resistance exercise experience (on average almost five years). For example, they were required to be able to bench press at least their body weight and be able to at least squat a weight 1.25 times their body weight. A non-linear periodized training program was followed by the subjects during the six-week supplementation period. This program was split into three two-week microcycles. Measurements were taken after each microcycle. After both the first and third microcycles, a significant group-$\times$-time interaction was found for bench press training volume; it increased more in the TMG group than in the placebo group. Remarkably, a significant group-$\times$-time interaction was also found for the second micro cycle—except the volume had increased more in the placebo group than in the TMG group. It's unclear what caused this. The results were also inconclusive for squat training volume. The placebo group fared better after the first microcycle, and the TMG group fared better after the third microcycle. A trend was also found for a better vertical jump in the TMG group compared to the placebo group. No significant group-$\times$-time interactions were found for upper body strength (1-RM bench press) and lower body (1-RM squat). The varying results between the microcycles make it difficult to draw conclusions. At best you could say that TMG might improve work volume and muscle capacity, but doesn't seem to affect maximum strength efforts.

In addition to the exercise variables, the body composition was also determined. A skin caliper was used for this. Body fat percentage and fat mass decreased significantly in the TMG group post-intervention compared to pre-intervention (-3.2 % and -2.9 kg, respectively). This effect wasn't seen in the placebo group (+0.2 % and +0.3 kg). Lean body mass also significantly increased in the TMG group post-intervention versus pre-intervention (+2.4 kg), but not in the placebo group (+0.3 kg). The cross sectional area of the thigh and upper arm were also measured. No differences were found between the groups. However, there was a significant increase in the cross sectional area of the upper arm in the TMG group post-intervention versus pre-intervention. These results suggest that TMG supplementation has the potential to improve body composition. This effect would be in line with what is seen in animal studies, which indeed showed that TMG decreases fat mass and increases muscle mass [299, 226, 225].

11.4 Safety

TMG can be found in the diet in small amounts (several hundred milligrams). These amounts aren't only considered as safe, but even as healthy and desirable. This is based on several studies that indicate an association between TMG intake and certain aspects of health. For example, epidemiological research suggests that choline and TMG intake reduce the risk of cancer [430]. Another study shows a negative correlation between choline and TMG intake and indicators of obesity, including total fat percentage [157]. Although no correlation was found between choline and TMG intake and the incidence of cardiovascular disease [369, 325], a negative correlation was found with risk factors of cardiovascular disease [369]. Choline and TMG intake are also associated with lower insulin resistance in a cohort of healthy adults (the effect being stronger in women than in men) [158].

Therapeutically, TMG has been used to treat homocystinuria for several decades. Homocystinuria is a disorder of methionine metabolism. A dosage of 3 g twice a day (for adults) is used for the treatment of homocystinuria. This dosage is higher than that used by athletes and is well tolerated. A side effect that occurs relatively often, however, is stomach and intestinal complaints. Splitting the dose into smaller doses spread throughout the day might help here.

Remarkably, there are a few cases described in the literature in which TMG supplementation led to cerebral edema in patients with homocystinuria [490, 125]. It's unknown what the mechanism of action for this possible very rare side effect is. It's assumed, however, that the cerebral edema in these rare cases was related to the disorder of methionine metabolism rather than TMG.

A few clinical trials have been performed to test the effect of TMG on certain liver disorders. In a randomized placebo-controlled trial, 20 g TMG per day was taken by patients that suffered from non-alcoholic fatty liver disease [2]. The study lasted for a year and a few patients reported gastrointestinal complaints. No further side effects were noted at this (very) high dosage. In another (small-scale) trial, no side effects were found after a year of TMG supplementation, also at 20 g daily, in patients with non-alcoholic steatohepatitis [1].

In the previously discussed trials (Section 11.3), employing a dosage of 2.5 g TMG daily, no side effects were reported. A dosage of 2.5 g daily is considered as completely safe in the literature.

11.5 Conclusion

Clinical studies suggest that TMG supplementation might improve body composition and performance in strength-intensive exercises. The precise mechanism that's responsible for this is unclear. TMG might affect glycolysis, gene expression and phosphorylation status of several proteins involved in muscle hypertrophy, and the stability of proteins involved in energy metabolism. In any case, TMG can act as a methyl donor for the remethylation of homocysteine. Additionally, it's classified as one of the most important organic osmolytes.

The dosage used for its potential ergogenic effect is 2.5 g daily. This dosage is considered safe and is generally well tolerated. It's possible that some people experience stomach or intestinal complaints from TMG supplementation; spreading the dose over the day might be a solution for this. Although TMG is found in foodstuffs, it's not realistic to

get several grams of it from the diet. The amount found in the diet is quite limited and a regular daily intake is estimated to be 100–300 mg.

12. Vitamin D

12.1 Introduction

In the 1930s, the benefits of UV exposure by athletes were well known among German researchers [80]. Russian researchers also reported back in 1938 that UV radiation exposure improved speed in the 100-meter dash in a small group of students [175]. The control group improved their times by only 1.7 %, whereas the group exposed to UV radiation improved it by 7.4 %. Nevertheless, it would take until 1952 before it was concluded that these effects were caused by improvements in vitamin D status. The German professor Hans Eric Ronge stated: *(...) the production of vitamin D (or of a related steroid) explains the success of UV-radiation with regards to physical performance (...)* [388]. A (severe) vitamin D deficiency is associated with proximal myopathy (muscle weakness of the proximal muscles) and myalgia (muscle pain).

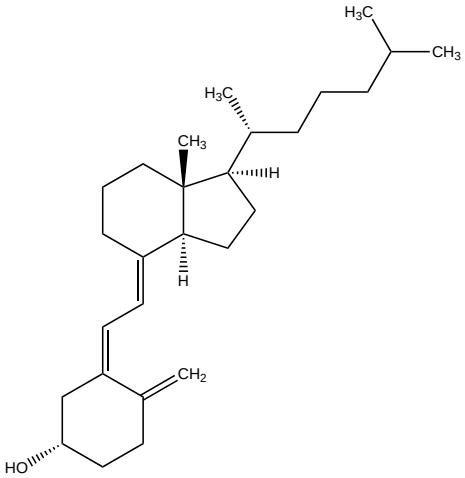


Figure 12.1: Structural formula of vitamin D3 (cholecalciferol).

Vitamin D can be produced by the human body under the influence of UV radiation. It can also be found in foodstuffs. However, it's not naturally found in many foods. Some exceptions to this are fatty fish such as salmon, mackerel and herring [218]. Nevertheless, the amounts in the diet are quite limited. A deficiency of the vitamin used to be very prevalent in the more northerly countries. This is the cause of the so-called English disease, also known as rickets, which causes skeletal abnormalities in children due to insufficient bone formation. (Vitamin D is tightly involved in calcium homeostasis.) The name of English disease can be traced back to nineteenth-century England. During this period, children were often put to work indoors. A result of which they were hardly exposed to sunlight—in the already cloudy and rainy country. To deal with this, vitamin D was added to common foods, especially dairy products such as milk and margarine. Nowadays, vitamin D fortification still occurs. In the Netherlands, it's mostly added to margarine and baking and frying products.

12.1.1 Biosynthesis

Vitamin D is sometimes referred to as a 'pseudo-vitamin'. After all, the human body can synthesize it. Vitamin D synthesis takes place in the skin on exposure to UVB radiation. Here, 7-dehydrocholesterol is photolyzed to previtamin D3. During this process, the B-ring of the steroid core 'snaps open'. The 7-DHC is mainly present in the epidermis where about two thirds of it resides. The remaining one third can be found in the dermis [220].

The formed previtamin D3 is then subjected to thermo-isomerization, in which a *cis* double bond is converted into a *trans* double bond. The product of this is called vitamin D3 (cholecalciferol).

The formed vitamin D3 then leaves the plasma membranes of the skin cells and reaches the bloodstream. In the bloodstream it will bind to vitamin D binding protein (DBP). However, vitamin D itself is not biologically active yet and has to be activated to its biologically active form. The bloodstream takes it to the liver, where it will undergo a hydroxylation reaction at carbon 25. This yields 25(OH)D3. This reaction is catalyzed by several enzymes belonging to the cytochrome P450 enzyme system. The enzyme CYP2R1 is likely to play the most important role in this [91]. 25(OH)D3 is then also transported in the bloodstream bound to DBP. Once it reaches the kidneys, it will undergo bioactivation to the bioactive hormone $1,25(OH)_2D3$. The enzyme responsible for this hydroxylation is CYP27B1 (also called 1α-hydroxylase) [43].

The degree to which previtamin D3 photosynthesis takes place depends on various factors, such as age, skin, pigmentation, and the degree to which the skin is exposed to UVB radiation. Older people have a significantly lower amount of 7-DHC in the skin [291], so that they synthesize less vitamin D3 when exposed to sunlight [221]. The majority (50–80 %) of the Dutch elderly population has been found to be vitamin D deficient [477]. Similar percentages are seen among Dutch Athletes [27]. About one third was found vitamin D deficient ($<$ 50 nmol/L 25(OH)D) and another one third was found to be vitamin D insufficient (50–75 nmol/L 25(OH)D) at the end of the winter. Skin color is also an important factor when it comes to the extent to which previtamin D3 photosynthesis takes place. Skin color is largely determined by the concentration of the skin pigment melanin. A higher concentration of melanin results in a darker skin. Because melanin is able to absorb UVB radiation, less 7-DHC ends up being photolyzed to previtamin D3. As a result, black people synthesize much less previtamin D3 [100]. This might possibly explain the high percentage of vitamin D deficiency encountered among Dutch immigrants

[477]. Of course, the degree to which the skin is exposed to UVB is also important. This is affected by wearing clothes, obviously, and how much someone is outside in the sun. Additionally, the application of sunscreen decreases vitamin D synthesis. Although its application has the potential to completely inhibit vitamin D synthesis [297], this isn't happening in practice. The thinner the layer of sunscreen that is applied, the more vitamin D that will be synthesized upon exposure to UVB radiation [149]. In practice, people often apply a layer that's thinner than recommended, and they don't repeat application often enough. Some spots are also missed when applying sunscreen. Finally, from October to March, it's almost impossible to synthesize previtamin D3 under the Dutch sun [474]. This period is also called 'vitamin D winter'.

Vitamine D status	Serum 25(OH)D concentration
Deficient	$<$ 50 nmol/l
Insufficient	52.5-72.5 nmol/l
Adequate	$\geq$75 nmol/l

Table 12.1: Interpretation of vitamin D status. Based on the Endocrine Society guidelines [219].

Further bioactivation of vitamin D3 to 1,25$(OH)_2$D is virtually unregulated when it comes to the first hydroxylation reaction (taking place at carbon 25). The serum level of 25(OH)D is thus a good reflection of vitamin D intake/synthesis. However, the subsequent α-oriented hydroxylation at carbon 1 is regulated. The three hormones parathyroid hormone (PTH), fibroblast growth factor 23 (FGF) and 1,25$(OH)_2$D itself are responsible for this. PTH is a hormone that's released by the parathyroid glands and stimulates, among other things, this final hydroxylation step required for the formation of 1,25$(OH)_2$D. The PTH secretion is regulated by the level of calcium in the circulation. Low calcium levels stimulate its secretion, while high levels inhibit it. FGF23 belongs to the phosphatonins, a group of hormones involved in phosphate homeostasis. In the kidney, FGF23 inhibits the activation of 25(OH)D to 1,25$(OH)_2$D and thus functions as a negative regulator.

12.1.2 Absorption

Vitamin D can be found in the diet in two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D2 is almost non-existent in the diet, with the exception of some mushrooms, food supplements and foods which are fortified with it. Vitamin D3, on the other hand, is found in several products of animal origin. Nevertheless, the amounts of vitamin D3 in these products are often quite low. Vitamin D3 can be found in reasonable amounts in fatty fish such as salmon, mackerel and herring [218]. The amounts found in these are 400–500 IU[1] per 100 g. With the current recommendation by the Netherlands Nutrition Centre of having (fatty) fish once a week, relatively little vitamin D comes from the diet. Moreover, most Dutch people aren't meeting this recommendation. Although baking and frying products can also be fortified with vitamin D (300 IU/100 g), this simply isn't sufficient either. As a result of the relatively low consumption of fish, most dietary vitamin D comes from fat and meat for many Dutch people [459].

When dietary vitamin D is consumed, it's usually together with fats. Mixed micelles

[1] 1 international unit of vitamin D is equal to 0.025 micrograms

are formed during the digestion of these fats. These are very small aggregates of various lipid molecules, such as phospholipids, fatty acids, and cholesterol. Vitamin D is also incorporated into these micelles [372]. In addition, a fraction is incorporated into lipid vesicles and possibly some of it binds with proteins. Uptake of this then takes place in the small intestine, in particular in the ileum and jejenum [374]. The amount that's absorbed is estimated to be 55–99 %, with an average of 78 % [374]. This absorption takes place both passively and carrier-mediated [375]. The transporters involved in this are cholesterol transporters.

Co-ingestion of vitamin D with fatty meals promotes its absorption [114]. Once in the enterocytes, a large part of the vitamin D is incorporated into chylomicrons, and thus it gets transportered via the lymphatic system together with other lipids before it reaches the circulation [61]. However, the small amount of 25(OH)D in the diet isn't incorporated into chylomicrons, but enters the liver via the portal vein. This is probably the result of its polarity and thus water solubility. Because vitamin D is transported to the circulation via the lymphatic system, it takes relatively long before a peak in the serum concentration will be reached. This is usually observed about ten to twelve hours after ingestion [61].

Of the two variants of vitamin D, vitamin D2 and vitamin D3, the latter is most effective in increasing the serum 25(OH)D concentration [449]. However, the absorption of both appears to be similar, so this effect is probably the result of differences in metabolism or clearance between the two [61].

12.1.3 Metabolism and excretion

Before vitamin D exerts its biological activity, it needs to be bioactivated. This bioactivation is achieved by virtue of two consecutive hydroxylation reactions. First, hydroxylation takes place at carbon 25, yielding 25(OH)D. This takes place in the liver. Several enzymes of the cytochrome P450 enzyme system are involved, including CYP27A1, CYP2J3, CYP2R1, and CYP3A4 [365]. CYP2R1 probably plays the most important role of these [91, 365, 7].

25(OH)D, bound to DBP, then enters the various tissues via the circulation, where it undergoes the final step for bioactivation: 1α-hydroxylation. This reaction is catalyzed by the CYP27B1 enzyme, yielding 1,25$(OH)_2$D. Most 1,25$(OH)_2$D is synthesized in the kidneys and is under the regulation of PTH, FGF23, and 1,25$(OH)_2$D itself. 1,25$(OH)_2$D is the major negative regulator of CYP27B1 activity. 1,25$(OH)_2$D achieves this by down-regulating the expression of the enzyme. It does so by binding to the vitamin D receptor (VDR) and subsequent direct and indirect interaction with vitamin D response elements [453].

It also seems that 1,25$(OH)_2$D stimulates the expression of FGF23 [496]. FGF23, in turn, down-regulates CYP27B1 expression, thus making this another way through which 1,25$(OH)_2$D regulates its own synthesis. FGF23 plays a central role in phosphate homeostasis. Secretion of this phosphatonin is also stimulated by increased phosphate levels, although the mechanism for this is uncertain.

Finally, PTH is able to stimulate CYP27B1 activity. PTH is primarily affected by calcium levels. Low calcium levels stimulates secretion of the hormone, while high levels inhibit it. PTH stimulates CYP27B1 expression by acting on the promoter region of the *CYP27B1* gene [64]. Thus, a low phosphate or calcium concentration stimulates CYP27B1 expression through FGF23 and PTH, respectively.

CYP27B1 is also expressed extrarenally (outside the kidneys) in many tissues [206]. The regulation of CYP27B1 in these tissues differs from that in the kidney. Additionally,

CYP27B1 mRNA and protein has been found in C2C12 myoblasts and myotubes [424]. CYP27B1 mRNA has also been detected in human muscle tissue [6]. Other research also found CYP27B1 mRNA in C2C12 myoblasts and myotubes, but failed to demonstrate $1,25(OH)_2D$ synthesis [455]. The extrarenal synthesis of $1,25(OH)_2D$, however, doesn't contribute to the serum concentration, although there are some exceptions to this (during pregnancy or in certain syndromes) [377].

Degradation of 25(OH)D and $1,25(OH)_2D$ starts with hydroxylation at carbon 24 or 23. The 24-hydroxylation is the first step in a series of reactions that breaks vitamin D down into more hydrophilic products. This series of reactions is catalyzed by the enzyme CYP24A1. The end product of this, starting with the hydroxylation of $1,25(OH)_2D$, is calcitroic acid [376]. This end product is very water-soluble and as such is cleared by the kidneys, ending up in the urine.

In addition to the metabolic pathway that starts off with 24-hydroxylation, CYP24A1 can also perform hydroxylation on carbon 23 as the first step [394]. The same factors that regulate CYP27B1—namely $1,25(OH)_2D$, PTH and FGF23—also regulate CYP24A1. However, the regulation by these factors is exactly opposite to that of CYP27B1. While $1,25(OH)_2D$ inhibits CYP27B1 activity, it actually stimulates CYP24A1 expression [244]. And while PTH is a stimulating factor for CYP27B1 activity, it inhibits CYP24A1 expression.

Finally, FGF23 also stimulates CYP24A1 expression, while down-regulating CYP27B1 expression. This opposing regulation of CYP24A1 versus CYP27B1 makes sense. While CYP27B1 increases the production of active vitamin D, CYP24A1 ensures that it's broken down and thus leads to a balance in the amount of $1,25(OH)_2D$. Glucuronidation of $1,25(OH)_2D$ can also take place [198]. This metabolite can be excreted in the bile, after which reabsorption from the small intestine can take place (enterohepatic cycle).

The half-life of 25(OH)D in serum is about fifteen days. The half-life of $1.25(OH)_2D$ is considerably shorter—ten to twenty hours [243]. Partly because of the long half-life, the serum 25(OH)D concentration is considered an important indicator of the vitamin D status [499].

12.2 Mechanism of action

Vitamin D is quite unique as a dietary supplement in regard to its mechanism of action. The reason for this is that it functions as a prohormone and therefore ultimately exerts its effects through a hormone receptor. In the case of vitamin D, this is the vitamin D receptor (VDR). The VDR is an intracellular receptor and belongs to the superfamily of nuclear receptors. It thus belongs to the same group of receptors as steroid receptors, such as the androgen and estrogen receptor. The VDR has a high affinity and selectivity for $1,25(OH)_2D$. After binding, the complex functions as a transcription factor that affects the expression of target genes. It does this by forming a dimer (a pair of two) with the retinoic acid receptor x (RXR). This VDR/RXR heterodimer recognizes specific parts of the DNA called vitamin D response elements. Subsequently, this complex, bound to a VDRE acts together with other proteins to stimulate or suppress the transcription of vitamin D target genes.

Which genes are regulated in a cell by vitamin D, and to what extent, is strongly dependent on the further cellular context. These effects, which are mediated by affecting the expression of genes by binding of vitamin D to the VDR, are called genomic effects.

In addition to these genomic effects, vitamin D also exerts so-called non-genomic effects. Important aspects in which these effects differ from genomic effects aren't only that they don't (directly) affect gene expression, but also that they proceed via completely different mechanisms and are rapid in nature. These non-genomic effects originate from the cell membrane instead of the cell nucleus. Nevertheless, the effect is probably still mediated by binding to the VDR, which appears to also be present in caveolea (bulb-shaped invaginations) of the cell membrane [201].

12.2.1 Classical effect on bone homeostasis

The classical role of vitamin D lies in the maintenance of calcium and phosphate homeostasis, making it essential for bone homeostasis. Bone consists mainly of calcium and phosphate in the form of hydroxyapatite crystals. When the serum calcium concentration decreases, the release of PTH increases. PTH then stimulates the activation of 25(OH)D in the kidneys by 1α-hydroxylation. Subsequently, $1,25(OH)_2D$ acts on the kidneys, bone and intestines to stabilize the serum calcium concentration. It enhances the reabsorption of Ca^{2+} in the kidneys. This limits the loss of calcium in the urine and thus helps to restore serum calcium levels. A similar effect of $1,25(OH)_2D$ is seen on the intestines, where it increases the absorption of calcium and phosphate.

At a 25(OH)D serum concentration of 86.5 nmol/L[2] the absorption of dietary calcium is 65 % higher compared to when the serum concentration is 50 nmol/L [204]. This enhancement is the result of both genomic and non-genomic effects.

Finally, $1,25(OH)_2D$ in conjunction with increased PTH levels stimulates the release of calcium (and phosphate) from bone tissue, in order to restore the serum calcium concentration. However, when calcium intake is adequate, the PTH levels won't be elevated, as a result of which vitamin D leads to healthy and strong bones.

Thus, calcium intake and vitamin D status both are important for bone homeostasis and preventing low bone density. As a consequence, vitamin D, along with calcium, is important for the prevention of bone fractures, including stress fractures. Stress fractures are relatively common among athletes, especially long-distance runners—often affecting the hips. Stress fractures are also common in military recruits, especially in women. In a double-blind, placebo-controlled trial, 5,201 female navy recruits were divided into two groups. One group received vitamin D (800 IU) and calcium (2,000 mg) daily, and the other group received a placebo. In the group receiving the supplement, the incidence of stress fractures was 20 % lower compared to the placebo group [274].

12.2.2 Non-genomic effects

Non-genomic effects are rapid in nature (requiring only seconds or minutes to take place) and often originate in the cell membrane. This is in contrast to genomic effects, which often take hours or sometimes even days to weeks to fully manifest. Genomic effects originate in the cell nucleus, where the DNA is located and gene transcription takes place. As of present, some non-genomic effects of vitamin D have been identified in muscle tissue. Nevertheless, it's still unclear which mechanisms actually play a significant role and to what extent. Moreover, most research has been conducted on birds and rodents.

A non-genomic effect that's in line with the role of vitamin D in calcium homeostasis is the regulation of intracellular Ca^{2+} in myoblasts and muscle cells [58]. Several studies

[2] 1 nmol/L 25(OH)D equals 0.40 ng/mL

demonstrate a rapid Ca^{2+} influx after exposure to vitamin D. This influx is at least partly caused by the effect of $1,25(OH)_2D$ on phospholipase C (PLC) and adenylyl cyclase activity. PLC cleaves phospholipids in the cell membrane, yielding diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 can then diffuse away from the cell membrane and bind to the IP3 receptor on the membrane of the sarcoplasmic reticulum. Activation of this receptor leads to a rapid Ca^{2+} efflux from the sarcoplasmic reticulum into the cytosol. Activation of adenylyl cyclase leads to the formation of the second messenger cyclic AMP (cAMP) from ATP. cAMP molecules then bind to protein kinase A (PKA) in order to activate it. The activity of L-type calcium channels is regulated by, among other factors, PKA [242]. The DAG that's formed also activates protein kinase C (PKC). This kinase enhances the influx of extracellular Ca^{2+} [82].

12.2.3 Activation of the Akt/mTOR pathway

Research examing the effect of $1,25(OH)_2D$ on anabolic pathways in muscle cells is scarce. One study investigated the combined effect of $1,25(OH)_2D$, insulin and leucine in C2C12 cells [396]. The C2C12 myoblasts were incubated with 1 or 10 nM $1,25(OH)_2D$ for 72 hours. After this, the cells were depleted of serum and leucine for 4 hours. Subsequently, the cells were treated for 30 minutes with insulin (100 nM) and leucine (5 nM). Subsequently, labeled valine was added (to determine the fractional protein synthesis rate) and several measurements were taken 50 minutes later. The incubation with 10 nM $1,25(OH)_2D$ together with leucine and insulin increased protein synthesis more than leucine and insulin alone. Several molecules involved in the Akt/mTOR pathway were examined: IR-P, Akt-P, mTOR-P, GSK3β-P, S6K1-P, 4EBP1-P, rpS6-P, and eEF2-P. There was a significant increase in IR-P, Akt-P, GSK3β-P, S6K1-P, and rpS6-P, but not in mTOR-P, 4EBP1-P, and eEF2-P when insulin and leucine were combined with $1,25(OH)_2D$. The mRNA and protein expression of the IR and VDR were also examined. Both increased by the incubation with $1,25(OH)_2D$. Taken together, this study suggests that $1,25(OH)_2D$ improves insulin signaling in C2C12 cells through increased expression of the IR and phosphorylation state of the IR and Akt.

Another study using human muscle cells showed that $1,25(OH)_2D$ inhibited the expression of protein phosphatase 2 (PPA2) [202]. PPA2 is a phosphatase that dephosphorylates Akt at Thr308 [270]. These results suggest that $1,25(OH)_2D$ might inhibit the dephosphorylation of Akt via inhibition of PPA2. Nevertheless, vitamin D supplementation doesn't seem to improve glycemic control in patients with type 2 diabetes [194, 269].

12.2.4 Inhibition of ubiquitin ligases

The ubiquitin system is responsible for the breakdown of proteins. It does so by tagging proteins with the molecule ubiquitin. A protein complex called the proteasome then recognizes these tagged proteins and breaks them down. Two major ubiquitin ligases that tag proteins for destruction are muscle atrophy F-box (MAFbx, also known as atrogin-1) and muscle ring finger 1 (MuRF1).

A recent study looked at the effect of vitamin D on the expression of these two ubiquitin ligases [202]. The researchers incubated human muscle cells with 10 nM $1,25(OH)_2D$ for 72 hours. Incubation resulted in a decrease in MAFbx and MuRF1 mRNA. IGF-1 expression was also measured, but no change was detected. In the same study, the effect of the cytokines tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) on the expression of the ubiquitin ligases was also examined. Both cytokines increased MAFbx

and MuRF1 expression, but this effect was inhibited by the addition of $1,25(OH)_2D$.

12.3 Clinical results

Many clinical studies that have evaluated the effects of vitamin D on muscle function were performed in the elderly or special populations. A 1979 trial showed an increase in the number of type 2 muscle fibers relative to type 1 muscle fibers in elderly patients with osteoporosis after supplementation of a vitamin D analogue combined with calcium [422]. The cross-sectional area of the type 2A muscle fibers also increased. A later placebo-controlled trial demonstrated that vitamin D supplementation increased the percentage and diameter of type 2 muscle fibers in older vitamin D deficient women [402]. Another study with older women showed similar results [87]. Older women were given vitamin D or a placebo. Those given vitamin D showed an increase in muscle fiber size compared to the placebo group (although this wasn't statistically significant).

However, the results of trials like these cannot simply be extrapolated to athletes. We therefore have to look at trials that specifically have examined the effects in athletes. Unfortunately, only a limited number of studies have done this. A small-scale, double-blind, placebo-controlled trial by Close et al. looked at the effect of vitamin D supplementation in ten professional soccer players [101]. Five of them were given vitamin D3 (5,000 IU daily) and five others were given a placebo, for a total of eight weeks. At baseline, the serum 25(OH)D concentration averaged about 30 nmol/L in the supplementation group, and 50 nmol/L in the placebo group. After 8 weeks, this had increased significantly to roughly 100 nmol/L in the supplementation group. The placebo group experienced a non-significant increase to around 70 nmol/L. The 1-RM bench press and squat were assessed in this trial. However, no significant group-$\times$-time interaction was found for these, although a trend was found ($P = 0.065$ and $P = 0.094$ for the 1-RM bench press and squat, respectively). Given the small sample size, it could be that the effect was too small to statistically detect (type 2 error). Nevertheless, a significant group-$\times$-interaction was found for the 10-meter dash and vertical jump height in favor of the supplementation group. Since the participants weren't following a training plan designed to increase strength, there simply might've not been an adequate stimulus to increase 1-RM lifts. Additionally, it has been suggested that a serum concentration of 125 nmol/L would be optimal for sports performance [80]. The serum concentration in the supplementation group didn't reach this concentration.

Later research, also by Close et al., was designed to investigate a dose-response relationship [102]. Soccer and football players affiliated with university athletics clubs were recruited. Ten of them were divided into a group that received 20,000 vitamin D3 per week, six 40,000 IU, and nine a placebo for a total of 12 weeks. The serum 25(OH)D concentration was approximately 50 nmol/L in each group. This increased to about 80 nmol/L in the 20,000 IU group and 90 nmol/L in the 40,000 IU group. A small decrease occurred in the placebo group, to about 40 nmol/L. Again, 1-RM bench press, 1-RM squat, vertical jump height and 20-meter dash were assessed. No significant effect was found between the groups for any of these variables. As in the previous study, the serum 25(OH)D concentration that was reached in the supplementation groups might not have been high enough for optimal sports performance. In addition, group sizes were quite small and no training routine aimed at strength development was followed during the supplementation period.

In a controlled trial by Wyon et al., elite classical ballet dancers could choose to receive

vitamin D3 ($n = 17$) or serve as a control group ($n = 7$) [488]. The supplementation period lasted four months, after which changes in strength, vertical jump height and injury frequency were compared between the two groups. As a measure of strength, a maximum isometric leg extension contraction for 5 seconds was used. A significant group-$\times$-time interaction was found for these three variables. The difference found in injury frequency was quite prominent. Only five injuries had occurred in the supplementation group, whereas seven occurred in the control group—while the supplementation group contained more subjects. The serum 25(OH)D values weren't measured in this trial, so it's unclear to what extent these values differed between the two groups and what concentration was reached by supplementation. Because this trial wasn't blinded and didn't have a placebo group, the level of evidence is moderate at best. As with the two studies discussed earlier, no training routine was followed for strength development.

Two other studies examined the effect of vitamin D2 instead of vitamin D3. A total of 33 high school athletes with vitamin D insufficiency (<75 nmol/L) were given 600 IU vitamin D2 daily ($n = 17$) or a placebo ($n = 16$) for 6 weeks [415]. In the supplementation group a significant but very small increase took place compared to the placebo group. No significant differences were found between both groups in terms of strength (assessed with a leg-back dynamometer), vertical jump height, and body fat percentage. The absence of an adequate training stimulus, the short supplementation period, low dosage and minimal difference in the serum 25(OH)D concentration between both groups could've masked a possible effect of vitamin D supplementation.

Other research, also using vitamin D2, gave 3,800 IU daily ($n = 13$) or a placebo ($n = 15$) to NASCAR pit crew athletes [329]. As with the previous study, the intervention period lasted six weeks and a leg-back dynamometer and vertical jump height were used as outcome measures. Furthermore, a Wingate test was performed and subjects had to bench press their body weight to exhaustion. Although the dose was much higher in this experiment, the total serum 25(OH)D concentration didn't increase. The subjects had a concentration of about 100 nmol/L at baseline. No significant differences were found between both groups.

In the context of improving muscle strength and body composition, a study combining vitamin D supplementation with resistance exercise and an appropriate diet in athletes would be interesting. However, such a clinical study is lacking to date. Nevertheless, such a study has been carried out in untrained young people and the elderly [6]. In this study, the young folks ($n = 20$) and the elderly ($n = 20$) were each divided into a vitamin D group and a placebo group. The vitamin D groups received 1,920 IU of vitamin D3 and 800 mg of calcium daily, whereas the placebo groups only received the calcium. The supplementation period lasted a total of sixteen weeks, of which the last twelve weeks included a resistance exercise program aimed at development of the quadriceps. Isometric knee extensions were performed to determine strength. The cross-sectional area of the quadriceps was also measured as a proxy for hypertrophy. The serum 25(OH)D concentration increased significantly in both vitamin D groups. It increased from 41.3 to 71.6 nmol/L in the young folks that received vitamin D, and from 70.7 to 111.2 nmol/L in the elderly. No significant effect of vitamin D supplementation on hypertrophy or strength was found.

12.4 Safety

Contrary to most dietary supplements, there's an abundance of data on the safety of vitamin D supplementation. Many review articles in the scientific literature are devoted to this topic [462, 199, 463, 243, 203, 116, 506, 400]. Too much vitamin D can lead to problems, mainly as a result of the hypercalcemia (too high calcium levels in the blood) that this can cause (and also hyperphosphatemia; too much phosphate in the blood). This can result in headache, nausea, vomiting, diarrhea, weight loss, polyuria and polydipsia. In the long term, mineralization of the soft tissues can start to take place, including calcification of the blood vessel walls. In any case, excessive activity of the vitamin D system is the culprit here [243].

The serum 25(OH)D concentration that can be achieved with an abundance of sunlight exposure is around 100–150 nmol/L [462, 35]. Vitamin D toxicity from sunlight exposure has never been reported in the literature, so it can be argued that this is a safe concentration. Indeed, the serum 25(OH)D concentration at which hypercalcemia starts to develop is uncertain, but it's estimated that it should be at least above 375–500 nmol/L for a prolonged period of time [243]. Such concentrations have only been observed in the literature in the event of an accidental overdose. The dose that would be required to reach a concentration above 100 nmol/L in most individuals appears to be around 4,000 IU daily [462, 464], assuming a low serum 25(OH)D concentration is present when starting supplementation (as usually is the case). In individuals that already have a concentration above 100 nmol/L, a further increase will occur by supplementation.

A risk analysis by Hathcock et al. states that the highest dose at which no adverse effects occur (NOAEL, no-observed-adverse-effect level) at 10,000 IU vitamin D3 daily [199]. This NOAEL is further supported by the absence of toxicity in clinical trials with healthy subjects which received dosages higher than this. However, the acceptable upper intake limit that is used by the Dutch Health Council is 4,000 IU daily. This number is based on the recommendation of the European Food Safety Authority (EFSA) [140].

There is also some evidence for non-hypercalcemic toxicity. For example, there are some studies that show an association between high serum 25(OH)D levels and, among other things, cardiovascular disease, cancer, allergies and mortality. However, most of these associations might be explained by several confounding variables. Nonetheless, the association with allergies appears probable [177]. However, the population in most of these studies which indicate this included infants and children. This makes it hard to extrapolate to adults. The underlying mechanism might be a shift in the balance between Th1 cells and Th2 cells towards more Th2 cells (see Box 12.1).

In any case, there's currently little reason to suspect harm of vitamin D supplementation at dosages lower than 10,000 IU vitamin D3 daily in healthy individuals.

Box 12.1

Th1 cells (T helper 1) and Th2 cells (T helper 2) are cell types of the immune system. Both are formed from the same precursor cell. Th1 cells are important in stimulating inflammatory responses by releasing the inflammatory factor tumor necrosis factor alpha (TNFα). They also activate macrophages through their interferon-gamma (IFN-γ) production. The primary role of these cells is therefore to neutralize intracellular pathogens such as viruses and some bacteria. The Th2 cells produce the interleukins IL-4, IL-5 and IL-13. This production stimulates the formation of so-called plasma cells. Plasma cells, in

turn, secrete various antibodies. When the balance between Th1 and Th2 cells shifts towards Th2 cells, an excess of antibodies (especially immunoglobulin E) can lead to allergy.

12.5 Conclusion

Vitamin D is produced by the skin under the influence of UVB radiation. Many Dutch people aren't exposed to enough sunlight during the summer to have produced enough vitamin D for the rest of the year. The dietary intake of vitamin D is also often insufficient to maintain adequate vitamin D levels. Recent research shows that vitamin D deficiency is common among Dutch athletes. Clinical research examining the effect of vitamin D supplementation on body composition or muscle strength in athletes is, unfortunately, limited, but suggests a possible positive effect on muscle strength. More adequate follow-up research is needed to confirm this.

It has been suggested that a serum 25(OH)D concentration of 125 nmol/L is required for optimal sports performance. However, more research is needed to determine whether this optimal concentration actually exists, and what the precise concentration would be. Depending on the individual's vitamin D status, dosages of up to 5,000 IU vitamin D3 daily might be required to achieve adequate levels. Some factors increase the risk of vitamin D deficiency or insufficiency, such as limited sunlight exposure, dark skin pigment, and older age. Certainty about the vitamin D status can be provided with a blood test measuring the 25(OH)D concentration. When a low concentration is found, supplementation is recommended. After three months of supplementation, another blood test can be taken to determine whether the dose needs to be adjusted.

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