PLANT PHYSIOLOGY AND BIOCHEMISTRY

Nitrogen and Lipid Metabolism

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Nitrogen fixation

Introduction

Nitrogen is the most important element, next to carbon, hydrogen and oxygen for sustaining life on earth. It is the most important component of biomolecules like proteins, nucleic acid, growth hormones, vitamins etc. One to fifteen percent of the dry weight of plant leaves is nitrogen. The productivity of the plants is determined by the availability of N\textsubscript{2} for its growth. 80% of the earth’s atmosphere is nitrogen, but it cannot be utilized as such. This is because the triple bounded structure dinitrogen, which is extremely stable and chemically unreactive. The atmospheric nitrogen is made available to the living world either by symbiotic or non-symbiotic means. The dinitrogen will be converted into nitrite (NO\textsubscript{2}\textsuperscript{-}), nitrate (NO\textsubscript{3}\textsuperscript{-}) or ammonia (NH\textsubscript{3}) before it is utilized by the plants.

The major sources of nitrogen are:
1. Atmospheric nitrogen (Molecular nitrogen) which can be utilized, only by few living organisms;
2. Nitrites and Nitrates of soil which will be converged into ammonia before entering the metabolic pathways;
3. Ammonium compounds of soil from which ammonium ions will be absorbed directly by the roots of plants along with the nitrate ions;
4. Organic nitrogen, present in the soil as proteins and partially destroyed proteins, which can be absorbed by the plants as amino acids, ammonia or nitrate directly.

In nature the process of nitrogen fixation will be either physical, chemical (industrial) or biological. The biological nitrogen fixation is more common in nature.

PROCESS OF NITROGEN FIXATION

Spontaneous or physical nitrogen fixation

It is a complex process accomplished in many steps, which starts with oxidation of atmospheric nitrogen under the influence of electric discharge during thunder. This will result in the production of nitric oxides.

\[
\text{Electric discharge and thunder: } \text{Nitrogen (N}_2\text{) + Oxygen (O}_2\text{)} \rightarrow \text{Nitric oxide (2NO)}
\]

The nitric oxide is again oxidised to nitrogen peroxide in presence of atmospheric oxygen:

\[
\text{Nitric Oxide (2NO) + Oxygen (O}_2\text{)} \rightarrow \text{Nitrogen peroxide (2NO}_2\text{)}
\]

The nitrogen peroxide combines with rain water to form nitrous acid and nitric acid which comes to ground along with the rain.

\[
\text{Nitrogen peroxide + Rain H}_2\text{O} \rightarrow \text{Nitrous acid + Nitric acid (2NO}_2\text{)}
\]

HNO\textsubscript{2} \quad HNO\textsubscript{3}

In the ground, the alkali radicals of soil react with nitric acid to produce nitrates and nitrates which are soluble in water and can be absorbed by the plants through their roots.

\[
\text{Ca or K Salts + HNO}_3 \rightarrow \text{Ca or K nitrates}
\]
1. **Industrial or Chemical N\textsubscript{2} fixation**

In industries atmospheric nitrogen is fixed by chemical processes for the production of N\textsubscript{2}-fertilizers. Today, this is a very sophisticated, capital intensive industry exclusively based on the conventional Haber-Bosch process (Catalytic reduction of N\textsubscript{2} to NH\textsubscript{3}, using hydrogen obtained from natural gas) which takes place at high temperature (400-600°C) and pressure (100-200 atmosphere).

\[
N_2 + 3H_2 \rightarrow 2NH_3
\]

2. **Biological nitrogen fixation**

Biological nitrogen fixation is the conversion of atmospheric nitrogen to ammonia (N\textsubscript{2} \rightarrow NH\textsubscript{3}) either by symbiotic or nonsymbiotic means. In nature, some bacteria are capable of nitrogen fixation and such bacteria are termed as “Diazotrophs”.

Recent estimate shows nearly 2.4×10\textsuperscript{8} tones of nitrogen is fixed by different means per annum. About 2/3 (two third) of this input is biological, one quarter is industrial and the remaining is spontaneous N\textsubscript{2}-fixation.

The biological nitrogen fixation may be categorized into following two types

(a) Asymbiotic N\textsubscript{2}-fixation

(b) Symbiotic N\textsubscript{2}-fixation

This classification is maintained to refer two different types of organisms involved in N\textsubscript{2}-fixation.

(a) **Asymbiotic N\textsubscript{2}-fixing organisms**

A range of prokaryotic organisms are capable of fixing nitrogen, they include:

- **Free living nitrogen fixing organisms**

<table>
<thead>
<tr>
<th>Archaebacteria</th>
<th>Methanogens</th>
<th>Methanococcus volate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eubacteria</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Heterotrophs</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Facultative anerobes</td>
<td>Clostridium pasteurianum</td>
<td></td>
</tr>
<tr>
<td>Microaerobes</td>
<td>Klebsiella pneumoniae</td>
<td></td>
</tr>
<tr>
<td>Aerobes</td>
<td>Azotobacter vinelandii</td>
<td></td>
</tr>
<tr>
<td>Autotrophs</td>
<td>Azospirillum lipoferum</td>
<td></td>
</tr>
<tr>
<td>Chemotrophic bacteria</td>
<td><em>Thiobacillus ferroxidans</em></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic bacteria</td>
<td><em>Rhodospirillum rubrum</em></td>
<td></td>
</tr>
<tr>
<td>Cynobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unicellular</td>
<td><em>Gloeotheco spp.</em></td>
<td></td>
</tr>
<tr>
<td>Filamentous</td>
<td><em>Oscillatoria spp.</em></td>
<td></td>
</tr>
<tr>
<td>Heterocystous</td>
<td><em>Anabaena, Nostoc spp</em></td>
<td></td>
</tr>
</tbody>
</table>
(b) **Symbiotic nitrogen fixing organisms**

<table>
<thead>
<tr>
<th>Name</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizobiaceae</td>
<td>Legumes and Parasponia</td>
</tr>
<tr>
<td><em>Azorhizobium</em></td>
<td>do</td>
</tr>
<tr>
<td><em>Bradyrhizobium</em></td>
<td>-do-</td>
</tr>
<tr>
<td><em>Photorhizobium</em></td>
<td>-do-</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>-do-</td>
</tr>
<tr>
<td><em>Sinorhizobium</em></td>
<td>-do-</td>
</tr>
</tbody>
</table>

**Actinomycetales**

*Frankia*

**Cyanobacteria** *(Nostoc, Anabaena etc)*

- Gunnera (angisperm)
- Macrozamia (gymnosperm)
- Cycas coralloid roots (gymnosperms)
- *Azolla* (pteridophyte)
- Blasia (bryophyta)
- Rhizalenia (diatom)
- Lichens Siphonochalina (spong)

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**The flow of nitrogen through the biosphere controlled by micro-organism**

The flow of nitrogen through the biosphere is referred to as the nitrogen cycle.

![Nitrogen Cycle Diagram](image-url)
Nitrogen cycle: It is a Bio Geo Chemical cycle in which different microorganisms control the cycling of nitrogen through the biosphere. The nitrogen cycle can be divided into following five major steps.

1. The initial step of the nitrogen cycle is the reduction of atmospheric nitrogen to ammonia by certain bacteria. This process of nitrogen fixation is catalyzed by the enzyme complex nitrogenase. The free living nitrogen fixing bacteria in the soil, such as Azotobacter and Clostridium, can fix atmospheric nitrogen, but the majority of biological nitrogen fixation is carried out by Cyanobacteria and symbiotic bacteria. The symbiotic bacteria infect the roots of certain plants like legumes and develop spherical root nodules. In these nodules, bacteria reduce N₂ to ammonia, which they release into the cytosol of the host plant. *Rhizobium* is the most common genus of symbiotic nitrogen fixing bacteria. It is estimated that nitrogen fixing micro-organisms reduce more than $10^{10}$ kg of nitrogen per year.

2. Ammonia can be utilized directly by most soil organisms and plants. The majority of ammonia in the soil, however, is oxidized to nitrite and then to nitrate by certain free-living soil bacteria by the process called nitrification. Nitrifying bacteria obtain their energy through these oxidation reactions by using ammonia as an electron donor. Bacteria of the genus *Nitrosomonas* primarily oxidize ammonia to nitrite, whereas members of another genus, *Nitrobacter*, convert nitrite to nitrate.

3. Many soil microorganisms reduce nitrate to atmospheric nitrogen under anaerobic conditions. These reduction reactions are known as denitrification.

4. Plants absorb nitrate and reduce it to ammonia, which is utilized in the synthesis of amino acids and other organic compounds containing nitrogen. Plants have the ability to synthesize all amino acids required for their cellular process. Animals, however, must ingest plants (or other animals or animal tissues) in order to obtain essential amino acid.

5. Degradation of plant and animal tissues upon death yield organic residues that are converted to ammonia by saprophytic bacteria (bacteria that decompose nonliving organic material). This conversion process is known as ammonification. Saprophytic bacteria utilize ammonia in their own biosynthetic processes but release excess ammonia into the environment, thereby providing a substrate for nitrification. The urea excreted by animals also provides ammonia utilized in nitrification.

Symbiotic Nitrogen fixation in Legumes

Symbiotic nitrogen fixation in legumes involves complex anatomical, morphological and biological interactions between the host plant and the invading micro-organisms.

It is generally agreed that nitrogen fixers, particularly legumes, contribute substantially more nitrogen to the soil pool than do free living bacteria. Typically a hectare of legume *Rhizobium* association will fix 25 to 60 kg of dinitrogen annually, while nonsymbiotic organisms fix less than 5 kg ha⁻¹ (Sprent and Sprent, 1990).

INFECTION AND NODULE DEVELOPMENT

This sequence of events begins with bacterial infection of the root and ends with the formation of mature nitrogen fixing nodules.

The process involves a sequence of multiple interactions between the bacteria and the host roots. In effect, the rhizobia and the roots of the prospective host plant establish a dialogue in the form of chemical messages passed between the two partners. Based on studies carried out primarily with *Glycine, Trifolium* and *Pisum*, as many as nine to ten separate developmental stages have been recognized (Sperent and Sprent, 1990).

The nodulation process includes.

1. Multiplication of the rhizobia, colonization of the rhizosphere, and attachment to epidermal and root hair cells.
2. Characteristic curling of the root hairs and invasion of the bacteria to form an infection thread.
3. Nodule initiation and development in the root cortex. This stage is concurrent with stage 1, Release of the bacteria from the infection thread and their differentiation as specialized nitrogen fixing cells.
Rhizobia colonize the soil in the vicinity of the root hair in response to signals sent out from the host root. The rhizobia in turn stimulate the root hair to curl, at the same time sending nitrogenic signals that stimulate cell division in the root cortex.

Rhizobia invade the root by digesting the root hair cell wall and forming an infection thread. The rhizobia continue to multiply as the infection thread elongates towards the root cortex.

The infection thread branches to penetrate numerous cortical cells and a visible evident nodule develops on the root.

**The Early Stage Colonization and Nodule initiation**

Rhizobia are free living, saprophytic soil bacteria. Their numbers in the soil are highly variable, from a few as zero or 10 to as many as $10^7$ gram$^{-1}$ of soil, depending upon the structure of the soil water content, and a variety of other factors. In the presence of host roots, the bacteria are encouraged to multiply and colonize the rhizosphere. The initial attraction of rhizobia to host roots appears to involve positive chemotaxis, or movement towards a chemical stimulant.

Chemotaxis is an important adaptive feature in micro-organisms generally. It allows the organism to detect nutrients and other chemicals that are either beneficial or required for their growth and reproduction. Roots are known to exude a variety of amino acids, sugars, and organic acids that may function as nutrients for rhizobia and other soil micro-organisms. Pea roots, for example exude an unusual amino acid, homoserine, which is a preferred source of carbon and nitrogen for the pea root symbiont, *Rhizobium leguminosarum* (biovar viciae). A large increase in the number of rhizobia on the pea root surface was found to correlate with the liberation of significant amount of homoserine (Egeraat, 1975).

Another group of chemical that have been implicated in attraction of rhizobia are the flavonoids. A wide variety of flavonoids have now been characterized in root exudates, many of which stimulate nodulation but some of which actually inhibit the process (Rolfe, 1988). It must be noted, however, that flavonoids are found in root exudates of many non –leguminous species as well and there is no direct evidence that flavonoids serve as chemotactic agents.
Once rhizobia have colonized the rhizosphere, they begin to synthesize morphogenic signal molecules called nodulation factor, or nod factor (Denarie and Cullimore, 1993). Nod factors are derivatives of chitin, a β-1-4 linked polymer of N-acetyl-D-glucosamine found in the cell wall of fungi and exoskeletons of insects. Nod factors are similar polymer except that a fatty acid replaces the acetyl group at one end of the molecule. Nod factors are consequently considered lipo-chito-oligosaccharides. Nod factor secreted into the soil solution by the rhizobia induce several significant changes in the growth and metabolism of the host roots as a prelude to rhizobial invasion of the root hair and subsequent nodule development. These changes include increased root hair production and the development of shorter, thicker roots. Stimulated by the nod factors to review their growth, the root hairs develop branching and curl the tip.

Structures of three common flavonoids implicated in rhizobia-host interactions are luteolin (flavone), naringenin (flavonon), and daidzein (isoflavone), released by the host root. The flavonoid interacts with the product of the bacterial nod D gene, leading to the induction of other nodulation genes.

Before actually invading the host, rhizobia also release mitogenic signals that stimulate localized cell divisions in the root cortex. These cell divisions form the primary nodule meristem, defining the region in which the nodule will eventually develop. A second center of cell division arises in the pericycle. Eventually these two masses of dividing cells will fuse to form the complete nodule.

The nature of the mitogenic signal is unknown, although there is some evidence that the plant hormone ethylene could be involved. Ethylene promotes root hair development in some plants and responses similar to those induced by nod factors can be mimicked by ethephone, a chemical that releases ethylene. More recent experiments have shown that ethylene has no role in root hair deformation induced by nod factors, but enzymes involved in the synthesis of ethylene are expressed in the region of the cortex where the primary nodule meristem arises (Heidstra et al., 1997). It has been suggested that, although ethylene normally inhibits cortical cell division, it could be involved in determining the position of the primary nodule meristem in the cortex.

Rhizobia-host specificity is probably determined when the rhizobia attach to the root hairs and must involve some form of recognition between symbiont and host. As a general principle, recognition between cells involves chemical linkage that form between unique molecules on cell surfaces. In case of rhizobia host interaction, recognition appears to involves two classes of molecules: lectins and complex polysaccharides. Lectins are small, non-enzymatic proteins synthesized by the host and have the particular ability to recognize and bind to specific complexes carbohydrates.
Individual legume species each produce different lectins with different sugar binding specificities. Lectins appear to recognize complex polysaccharides found on the surface of the potential symbiont. Although bacterial surface normally contain an array of complex extracellular polysaccharides, the synthesis of additional nodulation-specific extracellular polysaccharides is directed by bacterial genes that are activated in the presence of flavonoids in the host root exudates. Host range specificity would thus result from attachment of the *Rhizobium* to the host root hair because of specific lectin surface polysaccharide interactions.

Other experiments have indicated the involvement of a calcium binding protein, called rhicadhesin, located on the surface of the *rhizobial* cell (Smit, *et al.*, 1989). Rhicadhesin appears to be common to all *rhizobia* and is required for attachment. In addition to lectin and rhicadhesin, other physicochemical factors may also have a role in attachment.

**Invasion of the Root Hair and the Infection Thread**

In the second stage of nodulation, the bacterium must penetrate the host cell wall in order to enter the space between the wall and the plasma membrane. The preferred attachment site is the tip of the growing root hair. The root hair grows by tip growth and the colonies of attached rhizobia become entrapped in the tip of the root hair as it curls around. Some *rhizobia* release enzymes such as pectinase, hemicellulase, and cellulase, which degrade cell wall materials. These allow the bacteria to break the cell wall and gain access to the underlying plasma membrane.

Once the rhizobia reach the outer surface of the plasma membrane, tip growth of the root hair ceases and the cell membrane begins to invaginate, results in a tubular intrusion into the cell, the *infection thread*. This contains the invading rhizobia. The infection thread elongates, and the thread moves through the root hair cell. A thin layer of celluloid material is deposited on the inner surface of its membrane.

The infection thread continues to elongate until it reaches the base of the root hair cell. As the infection thread moves through the root hair into the cortex, the bacteria continue to multiply. When the thread reaches the developing nodule, it branches so that many individual cells in the young nodule become infected.

**The Release of Bacteria**

The final step in the infection process occurs when the bacteria are "released" into the host cells. Actually the membrane of the infection thread buds off to form small vesicles, each containing one or more individual bacteria. Shortly after release, the bacteria cease dividing, enlarge, and differentiate into specialized nitrogen fixing cells called bacteroids. The bacteroids remain surrounded by a membrane, called the peribacteroid membrane. Differentiation into a bacteroid is marked by a number of metabolic changes, including the synthesis of the enzymes and other factors that the organism requires for nitrogen fixation. The infection process continues throughout the life of the nodule. As the nodule increases in size due to the activity of the nodule meristem, bacteria continue to invade the new cells.

Also as the nodule enlarges and matures, vascular connections are established with the main vascular system of the root. These vascular connections serve to import photosynthetic carbon into the nodule and export fixed nitrogen from the nodule to the plant.
Schematic diagram of a cross section through a mature nodule. Vascular connections with the host plant provide for the exchange of carbon and nitrogen between the host and the microsymbiont.

**Nitrogenase Catalyzes the Reduction of Atmospheric Nitrogen to Ammonia**

The enzyme responsible for Nitrogen fixation is termed nitrogenase which carries out the following reaction:

\[ \text{N}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow 2\text{NH}_3 + \text{H}_2 \]

All nitrogenase enzymes contain a Fe protein that is made up to two oxygen sensitive subunits of approximately 62 kDa molecular weight and contain 4Fe and 4S\(^{2-}\) atoms per dimer. The molybdenum-containing part of the nitrogenase enzyme is termed the MoFe protein. Detailed structure of the Fe and MoFe proteins have recently been obtained by X-ray crystallography (Georgiadis et al., 1992; Kim & Rees, 1992) and have been discussed by Eady & Smith (1992).

**(b) The Mode of Action**

The enzyme complex nitrogenase catalyzes the eight electron reduction of atmospheric nitrogen to ammonia. This process requires NADH and ferredoxin and is accompanied by the hydrolysis of 16 molecules of ATP for molecule of \( \text{N}_2 \) that is reduced.

\[ \text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{ATP} + 16\text{H}_2\text{O} \xrightarrow{\text{Nitrogenase}} 2\text{NH}_3 + 16\text{ADP} + 16\text{Pi} \]

In the process of nitrogen fixation, NADH, the ultimate electron donor in this reaction transfers electrons to ferredoxin. Reduced ferredoxin transfers these electrons to the iron protein of nitrogenase. The reduced iron protein then binds ATP, which increases the reducing power of the protein and enables it to pass electrons one by one to the iron molybdenum protein of nitrogenase. Once the iron protein is oxidized, ATP is hydrolyzed to ADP and Pi. The iron molybdenum protein stores electrons until there is sufficient electrical potential for electron transfer to atmospheric nitrogen, which binds to this protein. It is likely that electron are transferred to atmospheric nitrogen in three steps that produce divide (HN=NH), hydrazine (H\(_2\)N–NH\(_2\)), and ammonia. Nitrogenase in strongly inhibited by ammonia and can function only where the cell requires reduced nitrogen.
Pathway for the transfer of electrons from NADH to N₂

Probable steps in the reduction of N₂ to NH₃. The reduction of nitrogen to ammonia by nitrogenase probably occurs with diimide (HN=NH) and hydrazine (H₂N–NH₂) as intermediates.

Proposed intermediates and dinuclear active site for N₂ reduction by nitrogenase enzyme-bound diimide and hydrazine as intermediates. Suggested bond in a figure are indicated in Å and shaded areas represent electron pairs which make protonated; for details see text (from Hardy et al., 1971).
In addition to the reduction of nitrogen, nitrogenase can also reduce a variety of other substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide (N≡N–N’)</td>
<td>N₂, N₂H₄, NH₃</td>
</tr>
<tr>
<td>Nitrous oxide (N≡N–O)</td>
<td>N₂</td>
</tr>
<tr>
<td>Cyanide (C≡N’)</td>
<td>CH₄, NH₃, CH₃NH₂</td>
</tr>
<tr>
<td>Alkyl cyanides (R–C≡N)</td>
<td>R–CH₃, NH₃</td>
</tr>
<tr>
<td>Cyanamide (N≡NH₂)</td>
<td>CH₄, NH₃, CH₃NH₂</td>
</tr>
<tr>
<td>*Acetylene (HC≡H)</td>
<td>H₂C≡CH₂ ethylene</td>
</tr>
<tr>
<td>Alkynes (R–C≡CH)</td>
<td>R–CH≡CH</td>
</tr>
<tr>
<td>Proton (H⁺)</td>
<td>H₂</td>
</tr>
</tbody>
</table>

*Among these substrates acetylene (ethyne), the conversion of which to ethylene is the basis of the most commonly used assay for nitrogen fixation. The ethylene formed by nitrogenase can be readily assayed using a simple gas chromatographic method.

**Genetics**

The genetics of nitrogen fixation have been extensively studied in *K. pneumoniae*. There are 20 genes (nif) involved in the assembly of nitrogenase. The synthesis of nitrogenase is repressed by the presence of combined nitrogen and by O₂. In *K. pneumoniae* the general nitrogen regulation system (ntr) is also involved. the product of the ntr A gene is a sigma factor (σ4) that is required for the recognition of the unusual promoters of the ntr and nif genes.

![Fig. The nitrogen fixation (nif) regulation of K. pneumoniae showing the roles of the gene products in nitrogen fixation. Gene designated (+) and (-) are regulatory gene (Gallon, 1992)](image-url)
**Rhizobium nodulation genes**

It is now thought that specific compounds exuded from the roots are able to switch on nodulation (nod) genes in the rhizobium bacteria. These compounds are flavones and isoflavones (Rolfe, 1988). The nodulating genes in *R. leguminosarum* (Economou et al., 1990).

The gene nod D, appears to be directly under the influence of flavones or isoflavones and to regulate the other genes of the nod complex, including the host specific nod E and nod F genes. Some rhizobia, e.g. *R. meliloti* have three copies of nod D which may vary in their flavonoid recognition in this way may regulate the host range, or other factors such as the number of nodules formed, or the time between inoculation and nodule formation. Each operon of the nod genes in preceded by a conserved DNA sequence the ‘nod box’, which is presumably regulated by the product of the nod D gene (Fisher and Long, 1992).

The development of nodules in elicited by specific lipo-oligosaccharide signal molecules released by the rhizobium bacteria. These molecules consist of a chito-oligosaccharide backbone of different chain length contain at the non reducing end a long chain unsaturated fatty acid (Downie, 1991; Schaltze *et al.*, 1992). The genes nod ABC are highly conserved in *Rhizobium* and the corresponding protein have been shown to induce root deformation, branching and cortical plant cell division (Dudley *et al.*, 1987; Banfalvi and Kondorosi, 1989). It has recently been confirmed that three genes are involved in the synthesis of acetylated glucosamine oligosaccharide signal molecules (John *et al.*, 1993).

**Nitrate reductase and its regulation**

Plants assimilate most of the nitrate absorbed by their roots into organic nitrogen compounds. The first step of this process is the reduction of nitrate in the cytosol (Oaks 1994). The enzyme nitrate reductase catalyzes this reaction.

\[
\text{NO}_3^- + \text{NAD (P)} + \text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{NAD (P)}^+ + \text{H}_2\text{O}
\]

Where NAD (P) H indicates NADH or NADPH. The most common form of nitrate reductase uses only NADH as an electron donor; another form of the enzyme that is found predominantly in nongreen tissues such as roots can use either NADH or NADPH (Warner and Kleinhofs 1992).

The nitrate reductase of higher plants are composed of two identical subunits, each containing three prosthetic groups FAD (flavin adenine dinucleotide), heme, and molybdenum complexed to an organic molecule called a pterin (Mendel and Stallmeyer 1995; Campbell 1999).

Nitrate reductase is the main molybdenum-containing protein in vegetative tissues, and one symptom of molybdenum deficiency is the accumulation of nitrate that results from diminished nitrate reductase activity.

Comparison of the amino acid sequences for nitrate reductase from several species with those of other well-characterized proteins that bind FAD, heme, or molybdenum has led to the three-domain model for nitrate reductase shown in Figure. The FAD binding domain accepts two electrons from NADH or NADPH. The electrons then pass through the heme domain to the molybdenum then pass through the heme domain to the molybdenum complex, where they are transferred to nitrate.
Figure A: model of the nitrate reductase dimer, illustrating the three binding domains whose polypeptide sequences are similar in eukaryotes: molybdenum complex (MoCo), heme, and FAD. The NADH binds at the FAD-binding region of each subunit and initiates a two-electron transfer from the carboxyl terminus, through each of the electron transfer components, to the amino (N) terminus. Nitrate is reduced at the molybdenum complex near the amino terminus. The polypeptide sequences of the hinge region are highly variable among species.

**Regulation of Nitrate Reductase**

Nitrate, light, and carbohydrates influence nitrate reductase at the transcription and translation levels (Sivasankar and Oaks 1996). In barley seedlings, nitrate reductase mRNA was detected approximately 40 minutes after addition of nitrate, and maximum levels were attained within 3 hours. In contrast to the rapid mRNA accumulation, there was a gradual linear increase in nitrate reductase activity, reflecting the slower synthesis of the protein.

In addition, the protein is subject to post-translational modification (involving a reversible phosphorylation) that is analogous to the regulation of sucrose phosphate synthase. Light, carbohydrate levels, and other environmental factors stimulate a protein phosphatase that dephosphorylates several serine residues on the nitrate reductase protein and thereby activates the enzyme. Operating in the reverse direction, darkness and Mg$^{2+}$ stimulate a protein kinase that phosphorylates the same serine residues, which then interact with a 14-3-3 inhibitor protein, and thereby inactivate nitrate reductase (Kaiser et al., 1999).
AMMONIA ASSIMILATION

Ammonia is generated via nitrogen fixation and nitrate reduction represses the N₂-ase activity. Diazotrophs incorporate NH₃, rapidly at a very low concentration, to prevent it from reaching repressive concentration. There are two principal routes by which NH₃ may be incorporated into organic form. NH₃ may be assimilated by the cooperative action of two GS and GOGAT enzymes. The GS-GOGAT system is considered the likely route by which NH₃ is assimilated.

![Ammonia Assimilation Diagram]

But the large quantities of ammonia can be synthesized by a number of secondary metabolic reactions.

1. The conversion of glycine into serine in the photo-respiratory carbon and nitrogen cycle.
2. The catabolism of the transport compounds, asparagine, arginine and ureides.
3. During normal amino acid metabolism, e.g. conversion of cystathionine into homocystain in methionine biosynthesis and threonine into 2-oxobutyrate in isoleucine biosynthesis. The enzyme phenylalanine ammonia lyase produces ammonia in the conversion of phenylalanine into cinnamate, the first reaction involved in the synthesis of lignin a major constituent of secondary cell walls.
4. Proteins are frequently hydrolyzed during the germination of seedlings or following leaf senescence. Prior to the synthesis of transport compounds, ammonia is liberated through the operation of glutamate dehydrogenase:

\[
\text{Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{2-oxoglutarate} + \text{NH}_3 + \text{NADH} + \text{H}^+ \]
Two enzymes glutamine synthetase and glutamate synthase operate in tandem to form the glutamate synthase cycle of ammonia assimilation.

The α-amino group of most amino acids comes from the α-amino group of glutamate by transamination. Glutamine, the other major nitrogen donor, contributes its side chain nitrogen in the biosynthesis of a wide range of important compounds.

Glutamate in synthesized from \( \text{NH}_4^+ \) and \( \alpha\)-ketoglutarate, a citric acid cycle intermediate by the action of glutamate dehydrogenase. This enzyme has already been encountered in the degradation of amino acids. NAD\(^+\) is the oxidant in catabolism. By contrast, NADPH is the reductant in biosynthesis.

\[
\text{NH}_4^+ + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{glutamate} + \text{NADP}^+ + \text{H}_2\text{O}
\]

Ammonia ion is incorporated into glutamine by the action of glutamine synthetase on glutamate. This amidation is drive by the hydrolysis of ATP.

The regulation of glutamine synthetase plays a critical role in controlling nitrogen metabolism.

Glutamate dehydrogenase and glutamine synthetase are present in all organisms. Most prokaryotes also contain glutamate synthase, which catalyzes the reductive amination of \( \alpha\)-ketoglutarate. The nitrogen donor in this reaction is glutamine, and so molecules of glutamate are formed.

\[
\alpha\text{-ketoglutarate} + \text{glutamine} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{glutamate} + \text{NADP}^+
\]
When NH$_4^+$ is limiting, most of the glutamate is made by the sequential action of glutamine synthetase and glutamate synthase.

\[
\text{NH}_4^+ + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{ATP} \rightarrow \text{glutamate} + \text{NADP}^+ + \text{ADP} + \text{Pi}
\]

Glutamine synthetase

There is now a substantial body of evidence that clearly demonstrated that glutamine synthetase (GS) is the sole port of entry into amino acids in higher plants (Lea et al., 1990, 1992; Lea, 1991). GS catalyzes the ATP-dependent conversion of glutamate into glutamine:

\[
\text{Glutamate} + \text{ammonia} + \text{ATP} \rightarrow \text{Glutamine} + \text{AMP} + \text{Pi}
\]

The enzyme is an octameric protein with a native molecular weight of 350-400 kDa and has a very high affinity for ammonia (Km = 3-5 M). Early investigations suggested that there were two isoenzymes of GS present in plants, are located in the cytoplasm and one in the chloroplast or plastid (McNally et al., 1983).

The enzyme glutamine synthetase has been studied in Phaseolus vulgaris and Pisum sativum.

Five genes coding for GS have been identified in P. vulgaris (Forde & Cullimore, 1989). The α gln-β and gln-γ genes encode the cytosolic α, β and γ polypeptides which are located in the cytoplasm. The fifth GS gene, gln-ε, has been identified in a genomic clone, but no evidence for the expression of the gene has yet been obtained. Three genes, termed GS1, GS3A and GS3B have been shown to code for cytosolic GS in Pisum sativum.
A summary of the induction of the four GS genes during the development of the nitrogen fixing nodule and the primary leaf are shown in Fig.

Fig. The genetic control of glutamine synthetase isoenzyme in nodules roots and leaves of *Phaseolus vulgaris* (orde and Cullimore, 1989).

**Glutamate synthase**

This enzyme catalyzes the reductant dependent conversion of glutamine and 2-oxoglutarate to yield two molecules of glutamate. The two different forms of glutamate synthase are present in higher plants; one utilizes NADH as a source of reductant and other ferredoxin:

Glutamine + 2-oxoglutarate + ferredoxin (reduced) → 2-glutamate + ferredoxin (oxidized)

(a) **Ferredoxin-dependent enzyme** first detected in pea leaves by Lea and Miflin, in 1974, is an iron sulfur flavoprotein, represent nearly 1% of the total protein content of leaves (Marquez et al., 1988). The enzyme is a large monomeric protein with a molecular weight of 140-160 k Da. Tissue fractionation studies have shown that ferredoxin dependent glutamate synthase is localized in the chloroplasts of leaves (Wallsgrove et al., 1982).

(b) **NADH-dependent enzyme**

In green leaves the activity of the NADH-dependent enzyme is low in comparison to ferredoxin dependent activity (Wallsgrove et al., 1982; Hecht et al., 1988). Enzyme activity has been detected in range of non-green tissues e.g. roots, cotyledons and tissue culture cells (Lea et al., 1992). The NADH-dependent enzyme appears to play a major in the ammonia assimilation in nitrogen fixing nodules (Robertson et al., 1975; Awonaike et al., 1981; Anderson et al., 1989; Chen et al., 1990). The enzyme is a monomer and has a high molecular weight in the region of 200-225 k Da (Anderson et al., 1989; Chen and Cullimore, 1989).
LIPID STRUCTURE AND FUNCTION

The lipids or lipoids are a heterogenous group of compounds related to fatty acids which include fats, oils, waxes and other related substances. These are oily or greasy hydrophobic organic substances, relatively insoluble in water and considerably soluble in organic solvents. The term lipid is however, sometimes used to refer “fat-like” substances which may not be actually related to fatty acids.

Structure of some common fats and fatty acids Fig. 1.

Chemically, fats are defined as the esters of glycerol and fatty acids or as the triglycerides of fatty acids.

ALCOHOLS

Alcohols present in lipid molecules may be saturated or unsaturated. The common saturated alcohols include glycerol, cholesterol and higher alcohols such as cetyl alcohol and myricyl alcohol.

Among the unsaturated alcohols found in fats include a number of pigments like phytol (a constituent of chlorophyll) and lycophyll (found is tomatoes as a purple pigment).

FATTY ACIDS

Fatty acids are long chain organic acids, usually having 4 to 30 carbon atoms. They have a single carbonyl group and a long, nonpolar hydrocarbon 'tail' which gives most lipids their hydrophobic and oily or greasy nature. Fatty acids do not occur in free state in the cells or tissues but are present in covalently bound form in different classes of lipids, because in free state its carboxyl group will be ionized. Fatty acids which occur in natural fats are usually monocarboxylic and contain an even number of C atoms as they are synthesized from 2 carbon containing units. These are usually straight chain derivatives. The chain may be saturated (containing only single bonds) or unsaturated [containing one or more double bond]. Some fatty acids may have hydroxyl group (s) in the chain (hydroxy or oxygenated fatty acids) and still others may possess ring structure (cyclic fatty acids). Fatty acids are stored as energy reserves (fat) through ester linkage with glycerol to form triglycerides. But more usually, it is linked to other groups to form either esters or amides.

1. Saturated fatty acids (General formula C\textsubscript{n}H\textsubscript{2n+1} COOH.)

Even numbered straight chain saturated fatty acids are found in both plants and animals. In addition, lipids from all sources contain small quantities of saturated fatty acids with an odd number of carbon atoms (C\textsubscript{3}}
through C₁₇). Generally, these fatty acids with odd number of carbon account for less than 1% of the total fatty acids.

Table 1.

<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>Systemic name</th>
<th>Carbon skeleton</th>
<th>Structure</th>
<th>Common source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>n-Butanoic</td>
<td>4 : 0</td>
<td>CH₃(CH₂)₄ COOH</td>
<td>Butter</td>
</tr>
<tr>
<td>Caproic</td>
<td>n-Hexanoic</td>
<td>6 : 0</td>
<td>CH₃(CH₂)₆ COOH</td>
<td>Coconut and palm oils</td>
</tr>
<tr>
<td>Caprylic</td>
<td>n-Octanoic</td>
<td>8 : 0</td>
<td>CH₃(CH₂)₈ COOH</td>
<td>Coconut and palm oils</td>
</tr>
<tr>
<td>Capric</td>
<td>n-Decanoic</td>
<td>10 : 0</td>
<td>CH₃(CH₂)₁₀ COOH</td>
<td>Coconut and palm oils</td>
</tr>
<tr>
<td>Lauric</td>
<td>n-Dodecanoic</td>
<td>12 : 0</td>
<td>CH₃(CH₂)₁₂ COOH</td>
<td>Laurel oil, spermaceti.</td>
</tr>
<tr>
<td>Myristic</td>
<td>n-Tetradecanoic</td>
<td>14 : 0</td>
<td>CH₃(CH₂)₁₂ COOH</td>
<td>Butter and wool fats</td>
</tr>
<tr>
<td>Palmitic</td>
<td>n-Hexadecanoic</td>
<td>16 : 0</td>
<td>CH₃(CH₂)₁₄ COOH</td>
<td>Animal and plant fats</td>
</tr>
<tr>
<td>Stearic</td>
<td>n-Octadecanoic</td>
<td>18 : 0</td>
<td>CH₃(CH₂)₁₆ COOH</td>
<td>Animal and plant fats</td>
</tr>
<tr>
<td>Arachidic</td>
<td>n-Eicosanoic</td>
<td>20 : 0</td>
<td>CH₃(CH₂)₁₈ COOH</td>
<td>Groundnut oil</td>
</tr>
<tr>
<td>Behenic</td>
<td>n-Docosanoic</td>
<td>22 : 0</td>
<td>CH₃(CH₂)₂₀ COOH</td>
<td>Ground nut oil</td>
</tr>
<tr>
<td>Lignoceric</td>
<td>n-Tetracosanoic</td>
<td>24 : 0</td>
<td>CH₃(CH₂)₂₀ COOH</td>
<td>Groundnut and Rapeseed oils</td>
</tr>
<tr>
<td>Cerotic</td>
<td>n-Hexacosanoic</td>
<td>26 : 0</td>
<td>CH₃(CH₂)₂₆ COOH</td>
<td>Wool fat</td>
</tr>
<tr>
<td>Montanic</td>
<td>n-Octacosanoic</td>
<td>28 : 0</td>
<td>CH₃(CH₂)₂₈ COOH</td>
<td>-</td>
</tr>
</tbody>
</table>

In addition to the straight chain fatty acids, a number of branched chain fatty acids having even or odd number of carbon atoms have been identified as major compounds of natural fats and oils.

Table 2

<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>Systemic name</th>
<th>No. of C atoms</th>
<th>Structure</th>
<th>Common source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopalmitic</td>
<td>Isohexadecanoic</td>
<td>16</td>
<td>CH₃&lt;sup&gt;3&lt;/sup&gt;CH(2)&lt;sub&gt;12&lt;/sub&gt;COOH</td>
<td>Wool fat</td>
</tr>
<tr>
<td>Anteiso-</td>
<td>palmitic</td>
<td>17</td>
<td>CH₃CH₂CH(CH₂)₁₂COOH</td>
<td>Wool fat</td>
</tr>
<tr>
<td>Tuberculoste-</td>
<td>aric</td>
<td>19</td>
<td>CH₃&lt;sup&gt;3&lt;/sup&gt;CH(CH₂)₁₀COOH</td>
<td>Bacteria</td>
</tr>
</tbody>
</table>

2. **Unsaturated fatty acids**

The unsaturated fatty acids are may be classified based on the degree of unsaturation.

A. Monoethenoid acids – These contain one double bond and conform to the general formula CₙH₂ₙ₋₁ COOH. The common example is oleic acid.

B. Diethenoid acids – Two double bonds; CₙH₂ₙ₋₃ COOH; Linoleic acid.

C. Triethenoid acids – Three double bonds; CₙH₂ₙ₋₅ COOH; Linolenic acid.

D. Tetraethenoid acids – Four double bonds; CₙH₂ₙ₋₇ COOH; Arachidonic acid.
Monoethenoid acids are commonly called as monounsaturated fatty acids (MUFAs) and the remaining unsaturated fatty acids are classified under polyunsaturated fatty acids (PUFAs). In most of the unsaturated fatty acids, there is a double bond (designated $\Delta^9$) between carbon atom 9 and 10. This is particularly true of the unsaturated fatty acids, commonly found in the plant world. If there are additional bonds, they usually occur between $\Delta^9$ and the methyl-terminal end of the chain. It may, however, be generalized that in mammals, polyunsaturated fatty acids can have up to 22 carbon atoms and 6 double bonds, but in plants these acids do not exceed 18 carbon atoms and 4 double bonds Table – 3.

A most unusual unsaturated fatty acid, Nemotinic acid, is excreted in the growth medium by a citrivorium mould. This fatty acid is unique in that it contains the single, double and triple C-C linkages. Nemotinic acid in one of the few naturally occuring compounds containing the allene group.

![Nemotinic acid](image)

**Nemotinic acid**

<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>Systematic name</th>
<th>Carbon skeleton</th>
<th>Structure</th>
<th>Common source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crotonic</td>
<td>2-butenoic</td>
<td>4:1;2</td>
<td>CH$_3$CH=CHCOOH</td>
<td>Croton oil</td>
</tr>
<tr>
<td>Myristoleic</td>
<td>9-tetradecenoic</td>
<td>14:1; 9</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH (CH$_3$)$_2$COOH</td>
<td>Pycnanthyus</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>9-hexadecenoic</td>
<td>16:1; 9</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH(CH$_3$)$_2$COOH</td>
<td>Animal and plant fats</td>
</tr>
<tr>
<td>Oleic</td>
<td>9-Octadecenoic</td>
<td>18:1; 9</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH(CH$_2$)$_3$COOH</td>
<td>Animal and plant fats</td>
</tr>
<tr>
<td>Vaccenic</td>
<td>11-Octadecenoic</td>
<td>18:1;11</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH(CH$_2$)$_3$COOH</td>
<td>Bacterial fat</td>
</tr>
<tr>
<td>Linoleic</td>
<td>9,12-octadecadienoic</td>
<td>18:2;9, 12</td>
<td>CH$_2$(CH$_2$)$_2$CH=CHCH$_2$CH=CH(CH$_2$)$_3$COOH</td>
<td>Linseed and cotton seed oils</td>
</tr>
<tr>
<td>Eleostearic</td>
<td>9,11,13-octadecatrienoic</td>
<td>18:3; 9,11,13</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH=CH=CH=CH(CH$_2$)$_3$COOH</td>
<td>Tung oil</td>
</tr>
<tr>
<td>Linolenic</td>
<td>9,12,15-Octadecatrienoic</td>
<td>18:3;9, 12,15</td>
<td>CH$_2$(CH$_2$)$_2$CH=CHCH$_2$CH=CHCH$_2$CH=CH(CH$_2$)$_3$COOH</td>
<td>Linseed oil</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>5,8,11,14-eicosatetraenoic</td>
<td>20:4,5, 8,11, 14</td>
<td>CH$_2$(CH$_2$)$_2$CH=CHCH$_2$CH=CHCH$_2$CH=CHC H=CH=CH(CH$_2$)$_3$COOH</td>
<td>Animal fat</td>
</tr>
<tr>
<td>Nervonic</td>
<td>15-tetracosenoic</td>
<td>24:1;15</td>
<td>CH$_2$(CH$_2$)$_2$CH=CH(CH$_2$)$_3$COOH</td>
<td></td>
</tr>
</tbody>
</table>

The acetylene group has been detected in a number of unsaturated fatty acids, found in higher plants and micro-organisms. For eg., santalbic acid, a major component of the seed oil of sandalwood contains 1 acetylene group as against 2 of nemotinic acid.

![Santalbic acid](image)

**Santalbic acid**
**Geometric isomerism**

On account of the presence of double bond(s), the unsaturated fatty acids exhibit geometric (or cis-trans) isomerism. Most unsaturated fatty acids are found as the unstable cis isomer rather than as the more stable trans isomer.

The hydrocarbon chain of saturated fatty acids like stearic acid has a zigzag configuration with the C–C bond forming a bond angle of 109°.

The zigzag line represents the most stable configuration of such carbon chains.

The double bond in the zigzag line is indicated by drawing an extra line in between the carbon atoms involved in the double bond formation. When a cis double bond is inserted (as in oleic acid), the molecule bends assuming the shape as shown below.

**Nonconjugated double bond system**

Some another structural peculiarity of naturally occurring poly-unsaturated fatty acids is the presence of a non-conjugated double bond system. It has a methylene group (–CH₂–) flanked by double bonds on both the sides.
as in linoleic, linolenic and arachidonic acids. The conjugated double-bond system is, however, rarely present. In it the methylene group is not found is between the double bonds which, henceforth, occur one after the other as in eleosteric acid. This acid has valuable properties as a drying oil since it polymerizes readily. These two double-bond system have different chemical reactivities.

\[
\text{Nonconjugated system} \\
\text{Conjugated system}
\]

Two double bond system

3. *Hydroxy or oxygenated fatty acids*

One such fatty acid, found in castor oil is ricinoleic acid (87%). It is a \(C_{18}\) acid with a double bond at \(C_9\) and a hydroxyl group (OH group) on \(C_{12}\).

\[
\text{Ricinoleic acid} \\
\text{(12-hydroxy octadeca-9-enoic acid)}
\]

Cerebronic acid, a \(C_{24}\) acid obtained from animal lipid, is another important hydroxy acid with a hydroxyl group (OH group) on \(C_2\).

\[
\text{Cerebronic acid} \\
\text{(2-hydroxytetraicosanoic acid)}
\]

A common oxygenated fatty acid, isolated from plants and bacterial lipids, is

9, 10-dihydroxystearic acid.

\[
\text{9,10-dihydroxystearic acid} \\
\text{(9,10-dihydroxyoctadecanoic acid)}
\]

Similarly, 9, 10-epoxystearic acid is isolated from rust spore lipids (20%).

\[
\text{9,10-epoxystearic acid} \\
\text{(9,10-epoxyoctadecanoic acid)}
\]

4. *Cyclic fatty acids*

These fatty acids occur very rarely in the plant and animal kingdom. Chaulmoogra oil, obtained from the plant *Hydnocarpus kurzil*, used in the treatment of leprosy, contain 2 such acids hydnocarpic and chaulmoogric. Chaulmoogric acid has a cyclopentenyl ring in its 18 carbon structure.
Lipids from the lactobacilli contain a fatty acid, *lactobacilli acid*, with a cyclopropyl group. This fatty acid may result from the addition of a methylene group across the double bond of vaccenic acid (18:1; 11).

Similarly, sterculic acid from plant sources has a comparable structure, with a suggested relationship to oleic acid. It may be derived from oleic acid by the addition of a methylene group across the double bond in a manner that the unsaturated nature is not altered, unlike the lactobacillic acid.

**FUNCTIONS OF LIPIDS**

1. **Food material**: - Lipids present in food is of high calorific value. One gram lipid produces 9.3 kilocalories of energy.
2. **Food reserve**: - Lipids are insoluble in aqueous solutions and hence can be stored readily in the body as a food reserve.
3. **Structural component**: - Lipid is an important constituent of the cell membrane.
4. **Heat insulation**: - The fats are characterized for their high insulating capacity. Great quantities of fat are deposited in the subcutaneous layers in aquatic mammals such as whale and in animals living in cold climates to prevent heat loss.
5. **Fatty acid absorption**: - Phospholipids play an important role in the absorption and transportation of fatty acids.
6. **Hormone synthesis**: - The sex hormones, adrenocorticoids, cholic acids and vitamin D are synthesized from cholesterol, a steroidal lipid.
7. **Vitamin carriers**: - Lipids act as carriers of natural fat soluble vitamins such as vitamin A, D and E.
8. **Blood cholesterol lowering**: - The saturated fatty acid stearic acid plays an important roll in lowering the blood cholesterol level.
9. **Antibiotic agent**: - Squalamine, a steroid from the blood of sharks, has been shown to be an antibiotic and antifungal agent of intense activity. This seems to explain why sharks rarely contract infections and almost never get cancer.

............................................................................................................
LIPID BIOSYNTHESIS

Biosynthesis of Fatty Acids

When fatty acid oxidation was found to occur by oxidative removal of successive two carbon (acetyl CoA) units, biochemists thought that the biosynthesis of fatty acids might proceed by simple reversal of the same enzymatic steps used in their oxidation. However, fatty acid synthesis occurs in an entirely different pathway, and is catalyzed by different sets of enzymes and takes place in different parts of the cell.

Fatty acid biosynthesis can be conveniently divided into de nova synthesis, where a small precursor molecule (usually the 2C acetyl group) is gradually lengthened by 2C units, which give rise to 16C and 18C products, followed by various modifications (fig. 1).

The source of carbon for fatty acid biosynthesis varies in different organisms. Most of the carbon for de nova fatty acid (and lipid) formation goes through the pyruvate pool. Pyruvate is the end product of glycolysis.

Although pyruvate is produced in the cytosol (where the animal fatty acid biosynthesis takes place), acetyl CoA is mainly generated from pyruvate, present in the mitochondria. Under conditions favoring fatty acid biosynthesis, pyruvate is transported into mitochondria and pyruvate dehydrogenase is activated. The acetyl CoA product is combined with oxalacetate to produce citrate which leaves the mitochondria via a tricarboxylate anion carrier. Back in the cytosol, acetyl CoA is produced by ATP; citrate lyase. The NADPH needed for the reductive steps of fatty acid synthetase comes from the cytosolic pentose phosphate pathway.

In plants, the acetyl-CoA needed for lipid synthesis comes ultimately from photosynthesis. How the acetyl-CoA needed for fatty acid formation is generated within plastids is actually generated is not clear. For some plants, all the enzymes of glycolysis (including pyruvate dehydrogenase) appear to be present in the plastid. For other plants, such as spinach, acetyl-CoA arrives via a circuitous route from the mitochondria. Thus, acetyl-CoA generated by mitochondrial pyruvate dehydrogenase is hydrolyzed to free acetate. In this unionized form acetic acid can easily cross membranes and thus the 2C substrate moves to the plastid where it is activated by acetyl-CoA synthetase. Some plants use both the methods. (Fig. 2a and 2b).
Acetyl-CoA transport into the cytosol

Acetyl-CoA serves as a key intermediate between lipid and carbohydrate metabolism. For the production of fatty acids, acetyl-CoA (which is produced in mitochondria) must first be transported across the organelle's membrane into the cytosol. Since acetyl-CoA itself cannot traverse the membrane, this transfer relies on the transport of the acetyl moiety as citrate (produced from acetyl-CoA and oxaloacetate). After citrate is transferred via the tricarboxylate transport system from mitochondria into the cytosol, it is cleaved by ATP-citrate lyase to produce acetyl-CoA by the following reaction.

\[
\text{Citrate + CoA + ATP} \xrightarrow{\text{ATP-citratelyase}} \text{Acetyl-CoA + oxaloacetate + ADP + Pi}
\]

\(\Delta G' = -3400\) cal/mol

Although carnitine has been assigned the role as a carrier of acetyl groups, as well as of fatty acids, current evidence supports the contention that citrate and not acetyl-carnitine is the principal source of cytosolic acetyl-CoA. The acetyl-CoA is now ready to serve as a substrate with the required amounts of ATP and NADPH to form palmitate.
Fig.3 Acetyl-CoA as a key intermediate between fat and carbohydrate metabolism
Arrows identify major routes of formation or utilization of acetyl-CoA Citrate serves as a carrier to transport acetyl units from the mitochondrion to the cytosol for fatty acid synthesis.

Production of Malonyl CoA : The initiation Phase

The production of malonyl-CoA is the initial committed step in the fatty acid synthesis. In 1961, Salih Vakil's observation that CO₂ greatly stimulates the incorporation of acetyl CoA into fatty acid structure was an important finding in the elucidation of this process. In fact, his studies revealed that acetyl-CoA must be converted, rather carboxylated, into malonyl-CoA prior to its utilization for fatty acid synthesis. This irreversible two step reaction is the committed step in fatty synthesis and, as would be expected, it is also the primary rate limiting reaction of the process. The reaction is catalyzed by the enzymes, acetyl-CoA carboxylase.

The acetyl-CoA carboxylase from bacteria is a multienzyme complex which consists of 3 separate, functional polypeptide subunits
Fig. 4. The acetyl-CoA carboxylase reaction

Note that the long, flexible, biotin arm carries the activated CO\(_2\) from the biotin carboxylase region to the trans-carboxylase active site as shown in the lower diagrams. The active enzyme in each case is dark shaded.

(Redrawn from Lehninger, Nelson and Cox, 1993)

(a) biotin carboxyl carrier protein, BCP (MW = 45,000) containing two identical subunits each of which has one mole of biotin as its prosthetic group, covalently bound is amide linkage to an α-amino group of a lysine residue,

(b) biotin carboxylase, BC(MW = 98,000), an enzyme with two identical subunits and which catalyzes carboxylation of the biotin unit in biotin carboxyl carrier protein in an ATP-dependent reaction, and

(c) transcarboxylase, TC(MW = 1,30,000), an enzyme with two pairs of subunits of molecular weight 35,000 and 30,000 respectively and which catalyzes the transfer of activated CO\(_2\) unit from carboxybiotin to acetyl-CoA, producing malonyl-CoA.

In yeast, higher plants and animals, the activities of all the three subunits are present is a single biotin – containing polypeptide chain (MW 2,20,000).
Malonyl-CoA is synthesized in two steps by the action of two enzymes, each of which employs the biotin carrier proteins as one substrate. The two steps are:

**First Step** :- The biotin carboxylase (BC) catalyzes carboxylation of biotin carboxyl carrier protein (BCP) to yield carboxybiotin carboxyl carrier proteins (BCP-COO–); the carboxyl group being derived from bicarbonate (HCO3–). This is an ATP- dependent reaction.

\[
\text{BCP} + \text{HCO}_3^- + \text{ATP} \rightarrow \text{BCP-COO}^- + \text{ADP} + \text{Pi}
\]

Eq. (1)

**Second Step** :- The transcarboxylase transfers the "bound" CO2 from BCP-COO– to acetyl CoA, forming malonyl-CoA and regenerating BCP.

\[
\text{BCP-COO}^- + \text{CH}_3\text{CO}-\text{SCoA} \rightarrow \text{BCP} + -\text{OOC}^-\text{CH}_2\text{CO}-\text{SCoA}
\]

Eq. (2)

The free energy of cleavage of carboxybiotin proteins, \(\Delta G^0 = 4.7\) Kcal/mole at pH 7.0 is sufficient to allow the compound to act as a carboxylating agent in reaction (2) as well as in other reactions with suitable acceptors. The exergonic nature of the cleavage also explains the requirement for ATP for formation of the carboxybiotin protein.

Thus, the substrates are bound to acetyl CoA carboxylase and products are released in a specific sequence (fig. 5). Acetyl-CoA carboxylase exemplifies a ping-pong reaction mechanism in which one or more products are released before all the substrates are bound.

![Fig. 5: The reaction sequence of acetyl-CoA carboxylase](image)

Note that these reactions are "CO2-fixation" processes in which inorganic CO2 is used, even by animals, to form organic compounds. The overall result of these two reactions would be the production of a mole of malonyl CoA by the addition of a mole of CO2 (actually as HCO3–) to a mole of acetyl-COA, the ATP mole providing energy for driving the reaction. The net equation then would be

\[
\text{CH}_3\text{CO}^-\text{SCoA} + \text{HCO}_3^- + \text{ATP} \rightarrow -\text{OOC}^-\text{CH}_2\text{CO}^-\text{SCoA} + \text{ADP} + \text{Pi}
\]

Acetyl-CoA carboxylase (Acetyl-CoA) (Malonyl-CoA)

The malonyl-CoA provides 14 out of 16 carbon atoms of palmitate.

This reaction is very similar to other biotin-dependent carboxylation reactions, such as those catalyzed by pyruvate carboxylase and propionyl-CoA carboxylase.

Acetyl-CoA carboxylase is also important because it is a regulatory step; citrate acts as an allosteric activator for the animal enzyme, but not in plant or microbial systems. The high degree of structural organization of the animal carboxylases, which are absent in their counterparts in plants, yeast and *Escherichia coli*, suggests a possible structural role, in addition to their known catalytic and regulatory functions. It could serve as an organizing matrix for a supramolecular (multienzyme) complex with other enzymes which take part in lipid biosynthesis.

**Intermediates in Fatty Acid synthesis and the ACP**

Vagelos (1964) discovered that the intermediates in fatty acid synthesis are linked to an acyl carrier protein, ACP (MW = #9,000). Specifically, the intermediates are attached to the sulphydryl (–SH) terminus of a
Phosphopantetheine group. In the degradation of fatty acids, this unit is part of the CoA; whereas in synthesis, it is attached to a serine residue of the ACP Fig. 6. This single polypeptide chain of 77 residues can be regarded as a giant prosthetic group a "macro-CoA". The molecules apparently contain no cysteine.

As isomerase and the other is a reductase

*Upper figure, the fatty acid binds to the prosthetic group by forming a thioester bond with the sulfhydryl group. In other words, the –SH group is the site of entry of malonyl groups during fatty acid synthesis.

**Fig.6 Phosphopantetheine unit of ACP and CoA**

**Acyl carrier protein (ACP)**

ACP found in *Escherichia coli* is a small protein (Relative molecular mass, Mr = 8,860) containing the prosthetic group 4′-phosphopantetheine (Pn), an intermediate in the synthesis of coenzyme A. The thioester bond that links ACP to the fatty acyl group has a high free energy of hydrolysis. And when this bond is broken, energy is released which makes the first reaction in fatty acid synthesis (i.e. condensation reaction) thermodynamically favorable. The 4′-phosphopantetheine prosthetic group of ACP serves as a flexible arm, tethering the growing fatty acyl chain to the surface of the fatty acid synthase complex and carrying the reaction intermediate from one enzyme active site to the other.

**The Fatty Acid Synthase Complex and Seven different Active Sites**

All of the reactions in the biosynthesis of fatty acids are catalyzed by a multienzyme complex, the fatty acid synthase. The detailed enzyme structure and its location in cell differ from one species to anther, but the reaction sequence is identical in all organisms. The fatty acid synthesizing system from 3 sources have been investigated in some detail that from yeast by Lynen (1952), with a particle molecular weight of $2.3 \times 10^6$; that from pigeon liver by Wakil (1961), with a molecular weight of $4.5 \times 10^5$, that from *Escherichia coli* by Vagelos (1964). Of these systems, that from *E. coli* is perhaps the best understood at present.

The fatty acid synthase system from *E. coli* consists of seven separate polypeptides that are tightly associated in a single, organized complex. (Table 1). The proteins act together to catalyze the formation of fatty acids from acetyl CoA and malonyl-CoA. Throughout the process, the intermediates remain covalently attached to one of the two thiol (–SH) groups of the complex. The growing fatty acid is shifted between these two –SH groups. One is relatively fixed in position because it is a cysteine residue. It acts as a parking place for acyl groups, which are to be lengthened. The other –SH group carries the extended chain while it undergoes the reaction necessary for reduction to a saturated acyl group, and it also accepts the acetyl and malonyl groups from which the fatty acid is built. This –SH group can swing across the 7 different catalytic sites because it is located in a residue of phosphopantetheine.
Table 1: Seven components* of the fatty acid synthase complex from *Escherichia coli.*

<table>
<thead>
<tr>
<th>Component</th>
<th>Abb.</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl carrier protein</td>
<td>ACP</td>
<td>Carries acyl groups in thioester linkages</td>
</tr>
<tr>
<td>Acyl-CoA-ACP transacylase</td>
<td>AT</td>
<td>Transfers acyl group from CoA to cysteine residues of KS</td>
</tr>
<tr>
<td>Malonyl-CoA-ACP transferase</td>
<td>MT</td>
<td>Transfers malonyl group from CoA to ACP</td>
</tr>
<tr>
<td>β-ketoacyl-ACP synthase</td>
<td>KS</td>
<td>Condenses acyl and malonyl groups</td>
</tr>
<tr>
<td>β-ketoacyl-ACP reductase</td>
<td>KR</td>
<td>Reduces β-keto group to β-hydroxy group</td>
</tr>
<tr>
<td>β-hydroxyacyl-ACP dehydrogenase</td>
<td>HD</td>
<td>Removes H₂O from β-hydroxyacyl-ACP, creating double bond.</td>
</tr>
<tr>
<td>Enoyl-ACP reductase</td>
<td>ER</td>
<td>Reduces double bond, forming saturated acyl ACP.</td>
</tr>
</tbody>
</table>

- ACP has the specific task of binding the acyl intermediates during fatty acid synthesis. Of these 7 components, ACP is not an enzyme while the remaining are enzymatic in behaviour.

The two thiol groups are designated as 'central' and 'peripheral'. The 'central' one is the –SH group of acyl carrier protein (ACP), with the intermediates of fatty acid synthesis form a thioester and the 'peripheral' one is the –SH group of a cysteine residue in β-ketoacyl-ACP synthase, one of the 7 proteins of the multienzyme complex.

Thus, the bacteria contain separate protein to catalyze the individual reactions of fatty acid synthesis, even the formation of malonyl-CoA occurs in 2 stages (carboxylation of biotin and transfer of the –COO– group to acetyl –CoA).

The seven active site a for fatty acid synthesis (6 enzymes + ACP) reside in seven active separate polypeptide in the fatty acid synthase of *Escherichia coli*; and it also holds good for the enzyme complex from higher plants (Fig. 7). In these, complexes, each enzyme is positioned with its active site near that of the preceding and succeeding enzymes of the sequence. The flexible pantetheine arm of ACP can reach all of the active sites, and it carries the growing fatty acyl chain from one site to the next; the intermediates are not released from the enzyme complex until the finished product is obtained.

Fig. 7 - A comparison among the fatty acid synthase complexes from different sources.

The point of interest that the fatty acid synthase from bacteria and plants is a complex where all seven reside in seven separate polypeptides. In yeast, all 7 activities reside is only 2 polypeptides. And in vertebrates the 7 activities reside in a single large polypeptide.
The fatty acid synthases of yeast and of vertebrates are also multienzyme complex, but their integration is even more complex than the same in *E. coli* and higher plants. In yeast, the seven distinct active sites reside in only two large, multifunctional polypeptides, and in vertebrates, a single large polypeptide. (Relative molecular mass, $M_r = 2,40,000$) contains all seven enzymatic activities as well as a hydrolytic activity that cleaves the fatty acid from the ACP like part of the enzyme complex. The active form of this multienzyme protein is a dimer ($M_r = 4,80,000$).

The organized structure of the fatty acid synthases of yeast and higher organisms enhances the efficiency of the overall process because of the following reasons:

1. The intermediates are directly transferred from one active site to the next.
2. The intermediates are not diluted in the cytosol.
3. The intermediates do not have to find each other by random diffusion.
4. The covalently bound intermediates are secluded and protected from competing reactions.
The Fatty Acid Synthesis by Acetyl-CoA and Malonyl CoA

Figure 8 shows the sequence of events that occurs during synthesis of a fatty acid.

Fig. 8. The sequence of events occurring during fatty acid synthesis

The fatty acid synthase complex is shown schematically. Each segment of the disc represents one of the 6 enzymatic activities of the complex: acetyl-CoA-ACP transacylase (AT); malonyl-CoA-ACP transferase (MT); β-ketoacyl-ACP synthase (KS), containing a critical Cys-SH residue; β-ketoacyl-ACP reductase (KR); β-hydroxyacyl-ACP dehydratase (HD); and enoyl-ACP reductase (ER). At the centre is an acyl carrier protein (ACP) with its phosphopantetheine arm (Pn) ending in another –SH.
Before the condensation reactions (the built up the fatty acid chain) can begin the two –SH groups on the enzyme complex must be charged with the correct acyl groups. The 'priming' of the system, as it is called, takes place in 2 steps.

In the first step, the acetyl group of acetyl CoA is transferred to the cysteine –SH group of the β-ketoacyl ACP synthase. This reaction is catalyzed by acetyl-CoA-ATP transacetylase. In the second step, the malonyl group from malonyl-CoA is transferred to the –SH group of ACP by the enzyme malonyl-CoA-ACP transferase, also part of the complex.

The Phase of Elongation

First Round: In the first round the charged synthase complex, the acetyl and malonyl groups are very close to each other and are activated for the chain lengthening process, which consists of the following four steps (or reactions).

1. Condensation

The first step in the formation of a fatty acid chain is condensation of the activated acetyl and malonyl groups to form an acetoacetyl group bound to ACP through the phosphopantetheine –SH group, thus producing acetoacetyl-ACP; simultaneously, a mole of CO₂ is eliminated from the malonyl group. In this reaction, catalyzed by β-ketoacyl-ACP synthase, the acetyl group is transferred from the cysteine–SH group of this enzyme to the malonyl group on the –SH of ACP, becoming the methyl terminal two carbon unit of the new acetoacetyl groups. The carbon atom in the CO₂ formed in this reaction is the same carbon atom that was originally introduced into malonyl-CoA from HCO₃⁻ by the acetyl-CoA carboxylase reaction. Thus, CO₂ is only transiently in covalent linkage during fatty acid biosynthesis; it is removed as such as each two carbon unit is inserted. Thus, the net effect of condensation reaction is the extension of the acyl chain by 2 carbon atom. Thus, the first condensation reaction in the biosynthesis of a fatty acid may be diagrammatically represented as in fig. 9.

By using activated malonyl groups in the synthesis of fatty acid and activated acetate in their degradation, the cell manages to make both processes favorable, although one is effectively the reversal of the other. The extra energy, needed to make fatty acid synthesis favorable, is provided by the ATP used to synthesize manoly-CoA from acetyl-CoA and HCO₃⁻. In effect, the condensation reaction is drived by ATP, although ATP does not directly participate in the condensation reaction. Rather, ATP is used to form an energy rich substrate in the carboxylation of acetyl-CoA to malonyl-CoA. The free energy stored in malonyl-CoA in the carboxylation reaction is released in the decarboxylation accompanying the formation of acetoacetyl-ACP. Although HCO₃⁻ is required for fatty acid synthesis, its carbon does not appear in the product. Rather, all of the carbon atoms of even-chain fatty acid are derived from acetyl-CoA.

The next 3 steps in fatty acid synthesis reduce the keto (–CO) group at C-3 to a methylene (–CH₂–) group, the result being the conversion of acetoacetyl –ACP into butyl –ACP.
2. Reduction of the Carboxyl group

The acetoacetyl group formed in the condensation step next undergoes reduction of the carboxyl group at C-3 to form D-β-hydroxybutyl-ACP. This reaction is catalyzed by β-ketoacyl-ACP reductase and the electron donor is NADPH. This reaction differs from the corresponding one in fatty acid degradation in two respects.

(a) The D-rather than the L-epimer is formed.
(b) NADPH is the reducing agent, whereas NAD⁺ is the oxidizing agent in β-oxidation. This difference exemplifies the general principle that NADPH is consumed in biosynthetic reaction, whereas NADH is generated in energy yielding reaction.

3. Dehydration

In the third step, the elements of water are removed from C-2 and C-3 of d-β-hydroxybutyryl-ACP to yield double bond in the product, trans-Δ²-butenoic-AKP (also called crotonyl-ACP). The enzyme that catalyzes this dehydration is β-hydroxyacyl-ACP dehydratase.

4. Reduction of the double bond

Finally, the double bond of trans-Δ²-butenoic-ACP is reduced is reduced (or saturated) to form butyryl-ACP by the enzymatic action of enoyl-ACP reductase; again NADPH is the electron donor on the reductant. The FAD⁺ is the oxidant in the corresponding reaction in β-oxidation.

These four reactions, taken together, complete the first round of elongation cycle. Thus, after the first round of elongation, the C₄ (butyl) precursor of palmitate has been synthesized from a C₂ (acetyl) and a C₃ (malonyl) unit, with the acetyl group constituting the two terminal carbons of the growing fatty acid chain (C₁₅ and C₁₆ in palmitate, for example).

The general sequence of condensation and reduction by fatty acid synthase may be schematically represented as:

\[
\text{Acyl group} + \text{malonyl gr.} \xrightarrow{\text{+NADPH}} \text{3-Ketoacyl gr.} \xrightarrow{\text{+NADPH}} \text{3-hydroxyacyl gr.} \xrightarrow{\text{H₂O}} \text{Enoyl gr.} + \text{NADPH} \xrightarrow{\text{Acyl group}} \text{Acyl group}
\]

Successive Rounds

The production of C-4 saturated fatty acyl-ACP (i.e., a C₄-butyryl-ACP) completes one round through the fatty acid synthase complex in fatty acid synthesis. During the second round of elongation phase, the butyryl group is now transferred from the phosphopantetheine –SH group of ACP to cysteine –SH group of β-ketoacyl-ACP synthase (KS). To start the next cycle of 4 reactions that lengthens the chain by 2 more carbons, another malonyl group is linked to the now vacant phosphopantetheine –SH group of ACP. Condensation occurs as the butyryl group, acting exactly as did the acetyl group in the first round, is linked to two carbons of the malonyl-ACP with simultaneous release of a mole of CO₂. The product of this condensation is a C-6 acyl group, covalently bound to the phosphopantetheine –SH group of ACP (i.e., a C₆-β-ketoacyl-ACP). Its β-keto group is reduced in the next 3 reaction of the second round of synthesis cycle to yield the C-6 saturated fatty acyl-ACP (i.e. a C₆-fatty acyl-ATP), exactly as in the first round of reactions.

The C₆-fatty acyl-ACP is now ready for a third round of elongation. Seven such cycles of condensation and reduction produce the C-16 saturated palmitoyl group still bound to ACP. This intermediate is not a substrate for the condensing enzyme, β-ketoacyl-ACP synthase (KS) and the chain elongation generally stops at this point. Rather, it is hydrolyzed to yield palmitate and ACP. Small amount of longer chain fatty acids such as stearate (18 : 0) are also formed. In certain plants (coconut and palm, for example), chain termination occurs earlier; a majority of the fatty acid (up to 90%) in the oils of these plants contain between 8 and 14 carbon atoms. Thus, we see that the fatty acid synthase reactions are repeated to form palmitate. The origin of carbon atoms in palmitic acid – Fig. 10.
**Stoichiometry of Fatty Acid Synthesis**

The overall reaction for the synthesis of palmitate from acetyl-CoA can be considered in two parts. **First, the formation of seven malonyl-CoA molecules**

\[
7 \text{Acetyl-CoA} + 7 \text{CO}_2 + 7 \text{ATP} \rightarrow 7 \text{malonyl-CoA} + 7 \text{ADP} + 7 \text{Pi} \quad \text{Eq. 1}
\]

**The seven cycles of condensation and reduction**

\[
\text{Acetyl-CoA} + 7 \text{malonyl-CoA} + 14 \text{NADPH} + 14 \text{H}^+ \rightarrow \text{palmitate} + 7 \text{CO}_2 + 8 \text{CoA} + 14 \text{NADP}^+ + 6 \text{H}_2\text{O} \quad \text{Eq. 2}
\]

**The overall process (the sum of Eq. 1 + Eq. 2) is**

\[
8 \text{Acetyl-CoA} + 7 \text{ATP} + 14 \text{NADPH} + 14 \text{H}^+ \rightarrow \text{palmitate} + 8 \text{CoA} + 6\text{H}_2\text{O} + 7\text{ADP} + 7\text{Pi} + 14\text{NADP}^+ \quad \text{Eq. 3}
\]

Note that the CO\(_2\) utilized (formation of malonyl–CoA) and the CO\(_2\) produced (condensation reaction) cancel each other when the overall stoichiometry is tabulated.

The biosynthesis of fatty acids such as palmitate, thus, requires acetyl–CoA and the input of chemical energy in 2 forms: the group transfer potential of ATP and the reducing power of NADPH. The ATP is required to attach CO\(_2\) to acetyl–CoA to produce malonyl –CoA; the NADPH is required to reduce the double bonds to form the corresponding saturated fatty acyl group.

**Acetate is shuttled out of Mitochondria in the form of Citrate**

In eukaryotes (non-photosynthetic), almost all the acetyl-CoA used in fatty acid synthesis is formed in mitochondria from pyruvate oxidation and from the catabolism of the carbon skeletons of amino acids. Acetyl-
CoA arising from the oxidation of fatty acids does not represent a significant source of acetyl-CoA for fatty acid biosynthesis in animals because the two pathways are regulated reciprocally.

The mitochondrial inner membrane is impermeable to acetyl-CoA, so an indirect shuttle transfers acetyl group equivalents across the inner membrane Fig. 11.

![Fig. 11. Shuttle for transfer of acetyl groups from mitochondria to the cytosol.](image)

**Fig. 11. Shuttle for transfer of acetyl groups from mitochondria to the cytosol.** The outer mitochondrial membrane is freely permeable to all of these compounds. Pyruvate derived from amino acid catabolism in the mitochondrial matrix, or from glucose by glycolysis in the cytosol, is converted to acetyl-CoA in the matrix. Acetyl groups pass out of the mitochondrion as citrate; in the cytosol they are delivered as acetyl-CoA for fatty acid synthesis. Oxaloacetate is reduced to malate, which returns to the mitochondrial matrix and is converted to oxaloacetate. An alternative fate for cytosolic malate is oxidation by malic enzyme to generate cytosolic NADPH; the pyruvate produced returns to the mitochondrial matrix.

Intra-mitochondrial acetyl-CoA first reacts with oxaloacetate to form citrate, in the citric acid cycle reaction catalyzed by citrate synthase. Citrate then passes into the cytosol through the mitochondrial inner membrane at the citrate transporter. In the cytosol, citrate is cleaved by citrate lyase regenerates acetyl-CoA in an ATP dependent reaction. Oxaloacetate cannot return to the mitochondrial matrix directly as there is no oxaloacetate transporter. Instead, oxaloacetate is reduced by cytosolic malate dehydrogenase to malate, which returns to the mitochondrial matrix on the malate-α-ketoglutarate transporter in exchange for citrate. There it is reoxidized to oxaloacetate to complete the shuttle.
Alternatively, the malate produced in the cytosol is used to generate cytosolic NADPH through the activity of malic enzyme Fig. 12.

![Diagram of NADPH produced by Malic enzyme](image)

**Fig. 12** NADPH produced by Malic enzyme

Palmitate is the precursor of stearate and longer chain saturated fatty acids, as well as the monounsaturated fatty acids, palmitoleate and oleate. Mammals can not convert oleate into linoleate or a linolenate, hence required in the diet as essential fatty acid (EFAs). Conversion of linoleate into other polyunsaturated fatty acids and eicoandnoids is also outlined.
Biosynthesis of Long-chain Fatty Acids

Palmitate (a C₁₆ fatty acid) is the major product of the fatty acid synthase system. This system is also called the de novo system in that the palmitate is constructed from acetyl-CoA (ACP) and malonyl-CoA (ACP). In plants and animals, the most important fatty acids are the C₁₈ fatty acids, namely stearic, oleic, linoleic and linolenic. These C₁₈ fatty acids, namely stearic, oleic, linoleic and linolenic are synthesized by elongation system which differ marked by from the de novo system. Palmitate acts as a precursor of other long chain fatty acids (Fig. 13). It may be lengthened to from stearate (18:0) or even larger saturate fatty acids by further addition of acetyl groups though the action of fatty acid elongation systems present in the endoplasmic reticulum (= microsomes) and mitochondria in the case of animals and in the soluble cytosol in the case of plants.

In animals

*Endoplasmic reticulum membrane*

\[
\text{Palmitoyl - CoA} \quad \text{Malonyl - CoA} + \text{NADPH} \quad \text{Stearyl - CoA}
\]

Mitochondrial outer and inner membrane

\[
\text{Palmitoyl - CoA} \quad \text{Acetyl - CoA} + \text{NADPH} \quad \text{Stearyl - CoA}
\]

In plants

Soluble cytosolic system

\[
\text{Palmitoyl -ACP} \quad \text{Malonyl - ACP} + \text{NADPH} \quad \text{Stearyl - ACP}
\]

Although different enzyme systems are involved and coenzyme A, rather than ACP, is the acyl carrier directly involved in animals fatty acid synthesis, the mechanism of elongation is otherwise identical with that employed in palmitate synthesis: donation of two carbons by malonyl ACP, followed by reduction (with NADH), dehydration and another reduction (with NADPH) to the saturated C₁₈ product, stearyl-CoA. Fasting largely abolishes chain elongation. Elongation of stearyl-CoA in brain increase rapidly during myelination in order to provide C₂₂ and C₂₄ fatty acids that are present in sphingolipids.

**BIOSYNTHESIS OF UNSATURATED FATTY ACIDS**

Palmitate and stearate serve as precursors of the two most common monounsaturated fatty acids of animal tissues, palmitoleate and oleate. Both of them possess a single cis double bond between C-9 and C-10. The double bond is introduced into the fatty acid chain by an oxidative reaction catalyzed by fatty acyl-CoA desaturase (Fig. 14). The enzyme is an example of a mixed function oxidase. Two different substrates, a fattyacyl-CoA and NADPH, simultaneously undergo two electron oxidations.
The pathway of electron transfer in the desaturation of fatty acids by a mixed function oxidase in vertebrates

[Two different substrates a fatty acyl-CoA and NADPH – undergoes oxidation by molecular oxygen. These reactions occur on the luminal face of the ER. A similar pathway, but with different electron carriers, exists in plants. The path of electron flow includes a cytochrome (cytochrome b\textsubscript{5}) and a flavoprotein (cytochrome b\textsubscript{5} reductase), both of which like fatty acyl – CoA desaturase itself are present in the smooth ER.]

Mammalian hepatocytes can readily introduce double bond at $\Delta^9$ position of fatty acids but cannot introduce additional double bond in the fatty acid chain between C-10 and the methyl terminal end. Thus, linoleate, 18 : 2 ($\Delta^{9,12}$) and $\alpha$-linolenate, 18 : 3 ($\Delta^{9,12,15}$) cannot be synthesized by mammals, but plants can synthesize both. The plant desaturases that introduce double bonds at $\Delta^{12}$ and $\Delta^{15}$ positions are located in the endoplasmic reticulum.

These enzymes, in fact, act not on free fatty acids but on a phospholipid called phosphotidylcholine which contains at least one oleate linked to glycerol (fig.15). Because linoleate and linolenate are necessary precursors for the synthesis of other products, they are essential fatty acids (EFA\textsubscript{s}) for mammals and must be obtained from plants material in the diet.
Fig. 15. Oxidation of phosphatidylcholine-bound oleate by desaturases, producing polyunsaturated fatty acids

Once ingested, linolenate, eicosatrienoate and eicosatetraenoate (=arachidonate), can be made only from linoleate. Arachidonate, 20: 4 (Δ5,8,11,14) is an essential precursor of regulatory lipids, the eicosanoids.

Families of Fatty Acids

Animal tissues contain a variety of polyunsaturated fatty acids. Of these, one series can be fabricated by the animal de novo. These are those fatty acids where all the double bonds lie between the 7th carbon from the terminal methyl group and the carbonyl group. Such fatty acids can be made by desaturation and chain elongation, starting with oleic acid (Fig. 13). Thus, oleate (which is produced from its corresponding saturated fatty acids, i.e., stearate) can be elongated to a 20: 1, cis- Δ11 fatty acid. Alternatively, a second double bond can be inserted to yield an 18: 2, cis – Δ6, Δ9 fatty acid. However, polyunsaturated fatty acids, in which one or more double bonds are situated within the terminal 7 carbon atoms, cannot be made de novo. Such polyunsaturated fatty acids are essential in the diet.
There are 4 series of polyunsaturated fatty acid in the mammals: two are derived from dietary linoleate and linolenate and the rest two are synthesized from the monounsaturated fatty acids, oleate and palmitoleate, which are formed from the corresponding saturated fatty acids. Conjugated double bonds are not formed in animal tissues. The four series can be recognized by the distance between the terminal methyl group (ω) and the nearest double bond. Desaturation and elongation reactions occur more extensively in liver than in extra hepatic tissue.

Fasting and diabetes are marked by an inhibition of desaturation pathways.

**The four series of polyunsaturated fatty acids**

<table>
<thead>
<tr>
<th>Precursor family</th>
<th>Systemic code</th>
<th>Formula</th>
<th>Series*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linolenate</td>
<td>18:3; 9, 12, 15</td>
<td>CH$_3$-(CH$_2$)$_4$-CH = CH-R</td>
<td>ω-3</td>
</tr>
<tr>
<td>Linoleate</td>
<td>18:2; 9, 12,</td>
<td>CH$_3$-(CH$_2$)$_3$-CH = CH-R</td>
<td>ω-6</td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>16:1; 9</td>
<td>CH$_3$-(CH$_2$)$_5$-CH = CH-R</td>
<td>ω-7</td>
</tr>
<tr>
<td>Oleate</td>
<td>18:1; 9</td>
<td>CH$_3$-(CH$_2$)$_7$-CH = CH-R</td>
<td>ω-9</td>
</tr>
</tbody>
</table>

* Note that the family of precursor and its products generated in animals through elongation and desaturation can be identified by subtracting the number designating the least double bond from the total number of carbon atoms. The result is the same within a family. For example, with linoleate, 18:2; 9, 12 and arachidionate, 20:4; 5, 8, 11, 14: 18-12 = 6 = 20-14.
**β-oxidation**

**DEGRADATION OF FATTY ACIDS**

The main pathways of fatty acid breakdown involve oxidation at various points on the acyl chain or lipoxidation at certain double bonds of specific unsaturated fatty acids.

The main forms of fatty acid oxidation are termed α, β and ω. They are named depending on which carbon on the acyl chain is attacked. β-oxidation is the most general and prevalent.

![Diagram of fatty acid oxidation](attachment:diagram.png)

**β-oxidation is the most common type of biological oxidation of fatty acids**

Long chain fatty acids, combined as triacylglycerols, provide the long term storage form of energy in the adipose tissues of animals. In addition, many plant seeds contain triacylglycerol stores. These fats are degraded principally by the liberation of 2C (acetyl – CoA) fragments in β-oxidation. The mechanism was originally proposed in 1904 by Knoop.

**Cellular site of β-oxidation**

Until relatively recently it had been considered that β-oxidation was confined to mitochondria. Although animal mitochondria do contain all the enzymes necessary and are a major site of β-oxidation, other subcellular sites, such as the microbodies, are implicated. Peroxisomes or glyoxysomes together are often referred to as microbodies. They contain a primitive respiratory chain where energy released in the reduction of oxygen is lost as heat. The presence of an active β-oxidation pathway in microbodies was first detected in glyoxysomes from germinating seed by de Duve in 1976.

Microbodies occur in all major groups of eukaryotes including yeasts, protozoa, plants and animals. The contribution of these organelles to total β-oxidation in a given tissues varies considerably. In animals microbodies are particularly important in liver and kidney. In liver it seems that mitochondria and microbodies collaborate in overall fatty acid oxidation. Thus, microbodies oxidize long-chain fatty acids to medium-chain products, which are then transported to mitochondria for complete breakdown. In this way very long chain fatty acids, such as erucate, which are poor substrates for mitochondria, can be catabolized.

In plants, the glyoxysomes from germinating seeds are capably of the complete breakdown of fatty acids to acetyl-CoA. They also integrate this metabolism with the operation of the glyoxylate cycle, which allows plants (in contrast to animals) to synthesize sugars from acetyl – CoA. Leaf tissues also contain peroxisomes and recent work indicates that β-oxidation in leaves is always found in peroxisomes with significant activity in mitochondria in some circumstances.

Mitochondrial β-oxidation of fatty acids takes place in three stages Fig -1. In the **first stage** of β-oxidation of fatty acids undergo oxidative removal of successive two carbon units in the form of acetyl-CoA, starting from the carboxyl end of the fatty acyl chain.
Fig. 1. Three stage of fatty acid oxidation

**Stage 1.** A long-chain fatty acid is oxidized to yield acetyl residues in the form of acetyl-CoA.

**Stage 2.** The acetyl residues are oxidized to CO$_2$ via the citric acid cycle.

**Stage 3.** Electrons derived from the oxidations of Stages 1 and 2 are passed to O$_2$ via the mitochondrial respiratory chain, providing the energy for ATP synthesis by oxidative phosphorylation.

*(Adapted from Lehninger, Nelson and Cox, 1993)*

In the **second stage** of fatty acid oxidation, the acetyl groups of acetyl-CoA are oxidized to CO$_2$ in the citric acid cycle, which also takes place in the mitochondrial matrix. Acetyl-CoA derived from fatty acids thus enters a final common pathway of oxidation along with acetyl-CoA derived from glucose via glycolysis and pyruvate oxidation.

In the **third stage**, the reduced electron carriers NADH and FADH$_2$, produced in the first two stages of oxidation donate electrons to the mitochondrial respiratory chain, through which the electron pass to oxygen. Coupled to this flow of electrons is the phosphorylation of ADP to ATP. Thus, energy released by fatty acid oxidation is conserved as ATP.
The Four Basic Steps of the β-oxidation of Saturated Fatty Acids

β-oxidation of saturated fatty acids is accomplished in 4-steps

![Diagram of β-oxidation](image)

**First step:** α, β dehydrogenation of acyl CoA

In first step, dehydrogenation of fatty acyl-CoA produces a double bond between the α and β carbon atoms (C-2 and C-3), yielding a trans-Δ2-enoyl-CoA (naturally occurring unsaturated fatty acids normally have their double bonds in the cis configuration).

The first step is catalyzed by three isozymes of acyl-CoA dehydrogenase. Long-chain acyl-CoA dehydrogenase (LCAD) acts on fatty acids of 12 to 18 carbons, and the isozymes specific for medium chain (MCAD) and short-chain (SCAD) fatty acids act on fatty acids of 4 to 14 and 4 to 8 carbons, respectively. These three acyl-CoA dehydrogenases are found in the matrix of mitochondria. They all have FAD as a prosthetic group. The electrons removed from the fatty acyl CoA are transferred to FAD, and the reduced form of the dehydrogenase immediately donates its electrons to an electron carrier of the mitochondrial respiratory chain, the electron transferring flavoprotein (ETF).

\[
\begin{align*}
\beta-
\end{align*}
\]

**ΔG = -4.8 kcal/mole**
The FADH$_2$ is not directly oxidized by oxygen but traces the following path.

![Diagram of electron transfer](image)

The oxidation catalyzed by acyl-CoA dehydrogenase is analogous to succinate dehydrogenation in the citric acid cycle; as in both the reaction:

(a) The enzyme is bound to the inner membrane.
(b) A double bond is introduced into a carboxylic acid between the $\alpha$ and $\beta$ carbons.
(c) FAD is the electron acceptor, and
(d) Electrons from the reaction ultimately enter the respiratory chain and are carried to $O_2$ with the concomitant synthesis of 2 ATP molecular per electron pair.

**Second step**: Hydration of $\alpha$, $\beta$-unsaturated acyl-CoAs.

In this step, a mole of water is added to the double bond of the trans-$\Delta^2$-enoyl-CoA to form the L-stereoisomer of $\beta$-hydroxyacyl-CoA (also called 3-hydroxyacyl-CoA). The reaction is catalyzed by enoyl-CoA hydratase or crotonase and has broad specificity with respect to the length of the acyl group. However, its activity decreases progressively with increasing chain length of the substrate (it may be noted that the enzyme will also hydrate $\alpha$, $\beta$-cis unsaturated acyl-CoA, but in this case D ($\gamma$)-$\beta$-hydroxyacyl-CoA is found).

$$\text{R–CH}_2–\text{CH=CH–C–S–CoA} + \text{H}_2\text{O} \xrightarrow{\Delta G'} = -0.75 \text{kcal/mole} \quad \text{R–CH}_2–\text{CH–CH–C–S–CoA}$$

This reaction catalyzed by enoyl-CoA hydratase is formally analogous to the fumarase reaction in the citric acid cycle, in which water adds across an $\alpha$–$\beta$ double bond. The hydration of enoyl-CoA is, in fact, the prelude to the second oxidation reaction, i.e., step 3.

**Third step**: Oxidation of $\beta$-hydroxyacyl-CoA.

In this step, the L-$\beta$ hydroxyacyl-CoA is dehydrogenated (or oxidized) to form $\beta$-ketoacyl-CoA by the action of an enzyme, $\beta$-hydroxyacyl-CoA dehydrogenase, which is absolutely specific for the L-stereoisomer of the hydroxyacyl substrate. NAD$^+$ is the electron acceptor in this reaction and the NADH, thus formed, donates its electrons to NADH dehydrogenase, an electron carrier of the respiratory chain. Three ATP molecules are generated from ADP per pair of electron passing from NADH to $O_2$ via respiratory chain.

$$\text{R–CH}_2–\text{CH=CH–C–S–CoA} + \text{NAD}^+ \xrightarrow{\Delta G} = +3.75 \text{kcal/mole} \quad \text{R–CH}_2–\text{CO–CH}_2–\text{C–S–CoA} + \text{NADH} + \text{H}^+$$

The reaction catalyzed by $\beta$-hydroxyacyl-CoA dehydrogenase is closely analogous to the malate dehydrogenase reaction of the citric acid. Thus, we see that the first three reactions in each round of fatty acid oxidation closely resemble the last steps in the citric acid cycle:

Acetyl-CoA $\rightarrow$ Enoyl-CoA $\rightarrow$ Hydroxyacyl-CoA $\rightarrow$ Ketoacyl-CoA
Succinate $\rightarrow$ Fumerate $\rightarrow$ Malate $\rightarrow$ Oxaloacetate

The net result of the first three reactions is the oxidation of a methylene group at $\beta$(or C-3) position to keep group of the substrate, acyl-CoA.

**Fourth step → Thiolysis or Thiolytic scission**

Thiolysis is the splitting by a thiol ($\sim$SH) group, aided by enzymatic catalysis. This final step brings about the cleavage of $\beta$-ketoacyl-CoA by the thiol group of a second mole of CoA, which yields acetyl-CoA, and an aryl-CoA, shortened by two carbon atoms. This thiolytic cleavage is catalyzed by the enzyme, acyl-CoA acyltransferase, which also have a broad specificity. This enzyme is more commonly called $\beta$-ketothiolase or simply thiolase.

\[
\frac{R-CH_2-CO-CH_2-C-S-CoA + CoA-SH}{\beta\text{-ketoacyl-CoA (n carbons) \enspace ΔG^\circ = -6.65 \text{ kcal/mole}}}{\frac{R-CH_2-CO-S-CoA + CH_3-C-CoA}{\text{Acetyl - CoA}}}
\]

Although the overall reaction is reversible, the equilibrium position is greatly in the direction of cleavage.

As to the mechanism of thiolase action, the enzyme protein has a reactive thiol (−SH) group on a cysteinyl residue that is involved in the following series of reaction.

\[
\frac{R-CH_2-CO-CH_2-C-S-CoA + Enz-SH}{\beta\text{-ketoacyl-CoA \enspace Thiolase}} \frac{R-CH_2-CO-S-Enz + CH_3-C-S-CoA}{\text{Acyl S-Enz \enspace Acetyl - CoA}}
\]

\[
\frac{R-CH_2-CO-S-Enz + CoA-SH}{\text{Acyl-CoA}} \frac{R-CH_2-CO-S-CoA + Enz-SH}{\text{Acyl-CoA}}
\]

The shortening of a fatty acyl-CoA derivative by two carbon atoms can be represented by the equation:

\[
R-CH_2-CH_2-CO-S-CoA + FAD + NAD + CoA-SH \rightarrow R-CH_2-CO-S-CoA + CH_3-CO-S-CoA + FADH_2 + NADH + H^+\]

The shortened acyl-CoA then undergoes another cycle of oxidation, starting with the reaction catalyzed by acyl-CoA dehydrogenase $\beta$-ketothiolase, hydroxyacyl dehydrogenase and enoyl-CoA hydratase all have broad specificity with respect to the length of the acyl group. Thus, by repeated turns of the cycle, a fatty acid is degraded to acetyl-CoA molecules with one being produced every turn until the last cycle, wherein two are produced. The $\beta$-oxidation of fatty acids is presented in a cyclic manner.
The β-oxidation cycle for fatty acid

The β-oxidation system is found in all organisms. However, in bacteria grown in the absence of fatty acids, the β-oxidative system is practically absent but is readily induced by the presence of fatty acids in the growth medium. The bacterial β-oxidation system is completely soluble and hence is not membrane bound. Curiously, in germinating seeds possessing high lipid content, the β-oxidation system is exclusively located in microbodies called glyoxysomes, but in seeds with low lipid content, the enzymes are seen within the mitochondria.

When a fatty acid pass through a β-oxidation sequence, one molecule of acetyl-CoA, two pairs of electron, and four protons (H⁺) are removed from the long-chain fatty acyl-CoA, shortening it by two carbon atoms. The fatty acid is repeatedly β-oxidated for its complete breakdown to acetyl-CoA, electrons, and protons.

Yield of ATP during oxidation of one Molecule of Palmitoyl-CoA to CO₂ and H₂O

<table>
<thead>
<tr>
<th>Enzyme catalyzing the oxidation step</th>
<th>Number of NADH or FADH₂ formed</th>
<th>Number of ATP ultimately formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA dehydrogenase</td>
<td>7FADH₂</td>
<td>10.5</td>
</tr>
<tr>
<td>β-hydroxyacyl-CoA dehydrogenase</td>
<td>7NADH</td>
<td>17.5</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>8NADH</td>
<td>20</td>
</tr>
<tr>
<td>α-ketoglutarate dehydrogenase</td>
<td>8NADH</td>
<td>20</td>
</tr>
<tr>
<td>Succinyl-CoA synthetase</td>
<td>GTP</td>
<td>8</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>8FADH₂</td>
<td>12</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>8NADH</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>108</td>
</tr>
</tbody>
</table>

*These calculations assume that mitochondrial oxidative phosphorylation produces 1.5 ATP per FADH₂ oxidized and 2.5 ATP per NADH oxidized. GTP produced directly in this step yields ATP in the reaction catalyzed by nucleoside diphosphate kinase.
OXIDATION OF UNSATURATED FATTY ACIDS

The fatty acid oxidation described earlier operates only when the incoming fatty acid is a saturated one (having only single bonds) and poses an even number of carbon atoms. However, most of the fatty acids in the triacylglycerols and phospholipids of animals and plants are unsaturated having one or more double bonds in its carbon chain. These bonds are in cis configuration and cannot be acted upon by the enzyme, enoyl-CoA hydratase which catalyzes the addition of H$_2$O to the trans double bond of the ∆-enoyl-CoA generated during β-oxidation. However, by the action of two auxiliary enzymes (an isomerase enzyme and a reductase enzyme), the fatty acid oxidation sequence described before can also break down the common unsaturated fatty acids.

(a) Oxidation of Monounsaturated Fatty Acids

The oxidation of monounsaturated fatty acids requires only one additional enzyme, enoyl-CoA isomerase. Oleate, an abundant C-18 monounsaturated fatty acid with a cis double bond between C-9 and C-10 (denoted cis-∆$^9$) is taken as an example. Oleate is converted into oleoyl-CoA which is transported through the mitochondrial membrane as oleoyl carnitine and then converted back into oleoyl-CoA in the matrix. Oleoyl-CoA then undergoes 3 passes through the β-oxidation cycle to yield 3 moles of acetyl-CoA and the coenzyme A ester of a ∆-12, 12-carbon unsaturated fatty acid, cis-∆$^3$-docosenoyl-CoA (Fig. 4). This product cannot be acted upon by the next enzyme of the β-oxidation pathway, i.e., enoyl-CoA hydratase, which acts only on trans double bonds. However, by the action of the auxiliary enzyme enoyl-CoA isomerase, the cis-∆$^3$-enoyl-CoA is isomerized to yield the trans-∆$^2$-enoyl-CoA. The latter compound is now converted by enoyl-CoA hydratase into the corresponding L-β-hydroxyacyl-CoA (trans-∆$^2$-docosenoyl-CoA). This intermediate is now acted upon by the remaining enzymes of β-oxidation to yield acetyl-CoA and C-10 saturated fatty acid as its coenzyme A ester (decanoyl-CoA). The latter undergoes 4 more passes through the pathway to yield altogether 9 (Nine) acetyl-CoAs from one mole of the C-18 oleate.

(b) Oxidation of Polyunsaturated Fatty Acids

This process requires two auxiliary enzymes, enoyl-CoA isomerase and 2,4-dienoyl-CoA-reductase. The mechanism is illustrated by taking linoleate, a C-18 polyunsaturated fatty acid with 2 cis double bonds at C$^9$ and C$^{12}$ (denoted cis-∆$^9$, cis-∆$^{12}$) as an example. Linoleoyl-CoA undergoes 3 passes through the typical β-oxidation sequence to yield 3 moles of acetyl-CoA and the coenzyme A ester of a C-12 unsaturated fatty acid with a cis-∆$^3$, cis-∆$^{12}$ configuration. This intermediate cannot be used by the enzymes of the β-oxidation pathway; its double bonds are in the wrong position and have the wrong configuration (cis, not trans).
However the combined action of enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase (Fig. 5) allows reentry of this intermediate into the typical β-oxidation pathway and its degradation to 6 acetyl-CoAs. The overall result is the conversion of linoleate to 9 moles of acetyl-CoA.

Here is an example of economy of organization of metabolism. The introduction of 2 additional types of enzymes (an enoyl-CoA isomerase and a 3-hydroxy acyl-CoA racemase) makes it possible to handle any combination of double bonds found in an unsaturated chain through the same route used for saturated fatty acids.

The roles of the 3 additional enzymes which are necessary for the oxidation of a dienoic (or polyenoic) acid may be shown in outline here, where A is enoyl-CoA isomerase, B, enoyl-CoA hydratase, and C, 3-hydroxyacyl-CoA epimerase, Monoenoic and dienoic acids are oxidized at comparable rates.

Fig. 5 The oxidation of polyunsaturated fatty acids requiring two additional enzymes, enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase. (Note that the combined action of these two enzymes converts a trans Δ2, cis Δ4-dienoyl-CoA intermediate into the trans Δ2-enoyl-CoA substrate, necessary for β-oxidation.)
OXIDATION OF ODD-CHAIN FATTY ACIDS

Complete oxidation of odd-number Fatty Acids requires three extra reactions. Although most naturally occurring lipids contain fatty acids with an even number of carbon atoms, yet fatty acids with an odd number of carbon atoms are found in significant amounts in the lipids of many plants and some marine animals. Small quantities of C-3 propionate are added as a mould inhibitory to some breads and cereals, and thus propionate enters the human diet. Besides, cattle and other remnants form large amounts of propionate during fermentation of carbohydrate in the rumen. The propionate so formed is absorbed into the blood and oxidized by the liver and other tissues.

A general scheme of the oxidation of an odd-chain fatty acid is presented in (Fig. 6).

The odd-carbon long chain fatty acids are oxidized by the same pathway as the even carbon fatty acids, starting at the carboxyl end of the chain. However, the substrate for the last pass through the β-oxidation cycle is a fatty acyl-CoA, in which the fatty acid has 5 carbon atoms. When this is oxidized and finally cleaved, the products are acetyl-CoA and propionyl-CoA, rather than 2 moles of acetyl-CoA produced in the normal β-oxidation cycle. The acetyl-CoA is, of course, oxidized via the citric acid cycle but the oxidation of propionyl-CoA presents an interesting problem, since at first glance the propionic acid (or propionyl-CoA) appears to be a substrate unsuitable for β-oxidation. However, the substrate is held by two strikingly dissimilar pathways: methylmalonate pathway and β-hydroxy propionate pathway.

Note the third remarkable reaction in which substituents on adjacent carbon atoms exchange positions; the coenzyme B₁₂ playing a key role in it.
Fig. 7. The methylmalonate pathway of propionate metabolism, as found in animals

This pathway is found only in animals and occurs in the mitochondria of liver, cardiac and skeletal muscles, kidney and other tissues. Propionate (or propionyl-CoA) is also produced by the oxidation of isoleucine, valine, methionine and threonine. Propionate is catalyzed by acetyl-CoA synthetase to produce propionyl-CoA (Fig. 7). The propionyl-CoA is carboxylated to from the D stereoisomer of methylmalonyl-CoA by and enzyme propionyl-CoA carboxylase, which contains the cofactors biotin. In this reaction, as in pyruvate carboxylase reaction, the CO$_2$ (or its hydrated ion, HCO$_3^-$) is activated by attachment to biotin before its transfer to the propionate moiety. The formation of the carboxybiotin intermediate requires energy, which is provided by the cleavage of ATP to AMP and PPi. The d-methylmalonyl-CoA, thus formed, is enzymatically epimerized to L-methylmalonyl-CoA, by the action of methylmalonyl-CoA epimerase (The epimerase labilizes the $\alpha$-hydrogen atom, followed by uptake of a proton from the medium, thus catalyzing interconversion of D- and L-methylmalonyl-CoA). The L-methylmalonyl-CoA undergoes an intramolecular rearrangement to form succinyl-CoA by the enzyme methylmalonyl-CoA mutase, which requires as its coenzyme deoxyadenosyl cobalamin or coenzyme B$_{12}$. When [2C$^{14}$] methyl-malonyl-CoA was converted by the mutase enzyme, the label was found in the 3 position of succinyl-CoA, thus indicating an intramolecular transfer of the entire thioester group, –CO-S-CoA, rather than migration of the carboxyl carbon.
The role of the coenzyme $B_{12}$ is to remove hydrogen from one carbon atom by transferring it directly to an adjacent carbon atom. The $H$ and $R$ are not released into solution.

\[
\begin{array}{c}
\begin{array}{c}
\text{H} \\
2 \text{C} \\
3 \text{C} \\
\end{array} \\
\begin{array}{c}
\text{R} \\
\end{array}
\end{array} \\
\begin{array}{c}
\begin{array}{c}
\text{R} \\
2 \text{C} \\
3 \text{C} \\
\end{array} \\
\begin{array}{c}
\text{H} \\
\end{array}
\end{array}
\]

At equilibrium, formation of succinyl-CoA favoured by a ratio of 20 : 1 over methyl-malonyl-CoA. The succinyl-CoA can then be oxidized via succinate and the citric acid cycle to $\text{CO}_2$ and $\text{H}_2\text{O}$. In patients with vitamin $B_{12}$ deficiency, both propionate and methylmalonate are excreted in the urine in abnormally large amounts.

The odd-chain fatty acids are only a small fraction of the total, and only the terminal 3 carbons appear as propionyl-CoA. The metabolism of propionyl-CoA is, therefore, not of quantitative significance in fatty acid oxidation. Two inheritable types of methylmalonic acidemia (and aciduria) are associated in young children with failure to grow and mental retardation.

In one type, the mutase protein is absent or defective since addition of coenzyme $B_{12}$ to liver extracts does not restore the activity of the mutase. In the other type, feeding large doses of vitamin $B_{12}$ relieves the acidemia and aciduria, and addition of coenzyme $B_{12}$ to liver extracts restores the activity of the mutase; in these cases, there is limited ability to convert the vitamin to the coenzyme.

Another inheritable disorder of propionate metabolism is due to a defect in propionyl-CoA carboxylase, resulting in propionic acidemia (and aciduria). Such individuals, as well as those with methylmalonic acidemia, are capable of oxidizing some propionate to $\text{CO}_2$, even in the absence of propionyl-CoA carboxylase.

### $\beta$-hydroxypropionate pathway

This pathway is ubiquitous in plants and is a modified form of $\beta$-oxidation scheme. It nicely resolves the problem of how plants can cope with propionic acid by a system not involving vitamin $B_{12}$ as cobamide coenzyme. Since plants have no $B_{12}$ functional enzyme, the methylmalonate pathway does not operate in them. This pathway, Fig. 8 thus, by passes the $B_{12}$ barrier in an effective way.
**Storage and Mobilization of fatty acids**

Many plants and animals need to store energy for use at a later. Lipid fuel stores are mainly triacylglycerols. Both animals and plants can store TAG in specialized tissues as to long term fuel reserves.

There are two types of adipose tissues in the animal body, known as 'brown' and 'white'. White adipose tissue is the more abundant and is the main tissue involved in the storage of body fat. Brown adipose tissue has a more specialized function in energy metabolism. White adipose tissue is widely distributed throughout the body. In human beings a large proportion is located just beneath the skin (subcutaneous adipose tissue) and is the tissue that influences the contours of the body. It also provides an insulating and protective layer. The adipose tissue is also located internally, for example surrounding the Kidneys (perirenal adipose tissue), along the intestinal tract (mesenteric adipose tissue) and in the omentum.

Although adipose tissue contains many types of cells, the ones responsible for fat storage are the adipocytes, which are bound together with connective tissue and supplied by an extensive network of blood vessels. Adipocytes are unusual in being able to expand to many times their original size by increasing their content of stored fat. The adipocytes (fat cells) are amorphous and widely distributed in the body, under the skin, around the deep blood vessels, and in the abdominal cavity. It typically makes up about 15% of the mass of a young adult human, with approximately 65% of this mass being in the form of triacylglycerols. Adipocytes are metabolically very active, responding quickly to hormonal stimuli in a metabolic interplay with the liver, skeletal muscles, and the heart. When the fat content of the diet is low, fat cells, can synthesize their lipid de
novo from glucose, which is transported into the cell from the bloodstream. When fat makes a large
contribution to dietary energy, the adipocytes can take in fat from circulating lipoproteins. This involves
hydrolytic breakdown of the TAG in the lipoproteins and release of fatty acids catalyzed by the enzyme
lipoprotein lipase. The fatty acids are transported into the cell and incorporated back into TAG.

Adipocytes just like other cell types in the body have an active glycolytic metabolism, they use the citric acid
cycle to oxidize pyruvate and fatty acids, and they carry out mitochondrial oxidative phosphorylation. During
period of high carbohydrate intake, adipose tissue can convert glucose via pyruvate and acetyl CoA into fatty
acids, from which triacylglycerols are made and stored as large fat globules. In humans, however, most fatty
acid synthesis occurs in hepatocytes, not in adipocytes. Adipocytes store triacylglycerols arriving from the
liver and from the intestinal tract, particularly after meals rich in fat.

When fuel is needed, triacylglycerols stored in adipose tissue are hydrolyzed by lipases within the adipocytes
to release free fatty acids, which may then be delivered via the bloodstream to skeletal muscles and the heart.
The release of fatty acids from adipocytes is greatly accelerated by the hormone epinephrine, which stimulates
the activation of triacylglycerol lipase. Insulin counterbalances this effect of epinephrine, decreasing the
activity of triacylglycerol lipase. Humans and many other animals, particularly those that hibernate, have
adipose tissue called brown fat, which is specialized to generate heat rather than ATP during oxidation of fatty
acids.

**Some plants store fats as minute globules in the seed**

There are hundreds of plants varieties known to have oil-bearing seeds, but only a few are significant
commercially. And most of them are important sources of edible oils for human foods or animal feeds, but
some are used for other industrial purposes such as paints, varnishes and lubricants. Although the seed is the
most important organ for the storage of triacylglycerols, some species stores large quantities of oil in the
mesocarp or pericarp of the fruit surrounding the seed kernel.

Microscopic examination of a mature oil seed or one in active phase of oil accumulation reveals a cytoplasm
packed with spherical organelles that consist mainly of triacylglycerols, called oil bodies. Other nomenclature
such as lipid bodies, storage oil bodies, storage oil droplets, oleosomes, lipid-containing vesicles and reserve
oil droplets, all of these are synonymous. Some authors have used the term spherosomes to refer to the same
organelle.

The core of the oil body is composed mainly of triacylglycerols with minor quantities of other hydrophobic
lipid such as sterols, hydrocarbons and carotenoids. The oil body is surrounded by a half-unit membrane of
protein and phospholipids.

In the animal kingdom, there is a close parallel to seed oil bodies, namely the cells of the brown adipose tissue
which are packed with small oil droplets. These cells also contain a large number of specialized mitochondria,
adapted for oxidizing the fatty acids from the oil droplets that they surround. Similarly, seed oil bodies are
surrounded by glyoxysomes. During seed germination these receive fatty acids from the oil bodies for
oxidation prior to the synthesis of carbohydrates, which occurs actively at this time.

Mobilization of fatty acids from the fat stores is regulated by hormonal balance, which in turn is responsive to
nutritional and physiological states

When hormones signal the seed for metabolic energy, triacylglycerols stored in adipose tissue are mobilized
(brought out of storage) and transported to tissues (skeletal muscle, heart, and renal cortex) in which fatty acids
can be oxidized for energy production. The hormones epinephrine and glucagon, secreted in response to low
blood glucose levels, activate the enzyme adenyl cyclase in the adipocyte plasma membrane Fig. 1, which
produces an intracellular secondary messenger, cyclic AMP (cAMP). A cAMP-dependent protein kinase
phosphorylates and thereby activates hormone sensitive triacylglycerols lipase, which catalyzes hydrolysis of the
ester linkage s of triacylglycerols. The fatty acids thus released pass from the adipocyte into the blood,
where they bind to the blood protein serum albumin. This protein (M, 68,000), which makes up about half of
the total serum protein, noncovalently binds as many as 10 fatty acids per protein monomer. Bound to this
soluble protein, the otherwise insoluble fatty acids are carried to tissues such as skeletal muscle, heart and
renal cortex. Here, fatty acids dissociate from albumin and are transported into cells to serve as fuel.
Low levels of glucose in the blood trigger the mobilization of triacylglycerols through the action of epinephrine and glucagon on the adipocyte adenyl cyclase. The subsequent steps in mobilization are described in the text.

About 95% of the biologically available energy of triacylglycerols resides in their 3 long-chain fatty acids; only five percent is contributed by glycerol moiety. The glycerol released by lipase action is phosphorylated by glycerol kinase (fig. 2), and the resulting glycerol-3 phosphate is oxidized to dihydroxyacetone phosphate. The glycolytic enzyme triose phosphate isomerase converts this compound to glyceraldehyde-3-phosphate, which is oxidized via glycolysis.
Fig. 2. Entry of glycerol into the glycolytic pathway

Activation of fatty acids and its transport to Mitochondria

The fatty acid oxidation enzymes in animal cells are located in the mitochondrial matrix, as demonstrated in 1948 by Eugene P. Kennedy and Albert Lehninger. The free fatty acid that enters the cytosol from the blood cannot pass directly through the mitochondrial membranes, but must first undergo a series of three enzymatic reactions. The first is catalyzed by a family of isozyme present in the outer mitochondrial membrane, the acyl CoA synthetases, which promote the general reaction.

The different acyl-CoA synthetase isozymes are specific for fatty acids having short, intermediate, or long carbon chain. Acyl-CoA synthetases catalyze the formation of a thioester linkage between the fatty acid carboxyl group and the thiol group of coenzyme A to yield a fatty acyl-CoA, coupled to the cleavage of ATP to AMP and PPI. The reaction occurs in two steps and involves a fatty acyl adenylate intermediate Fig.3.

\[
\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{Fatty acyl–CoA} + \text{AMP} + \text{PPI}
\]
Fig. 3. Conversion of a fatty acid to a fatty acyl-CoA

The conversion is catalyzed by fatty acyl-CoA synthetase and inorganic pyrophosphatase. Fatty acid activation by formation of the fatty acyl-CoA derivative occurs in two steps. First, the carboxylate ion displaces the outer two (β and γ) phosphates of ATP to form a fatty acyl-adenylate, the mixed anhydride of a carboxylic acid and a phosphoric acid. The other product is PP$_i$, an excellent leaving group that is immediately hydrolyzed to two P$_i$, pulling the reaction in the forward direction. The thiol group of coenzyme A carries out nucleophilic attack on the enzyme-bound mixed anhydride, displacing AMP and forming the thioester fatty acyl-CoA. The overall reaction is highly exergonic.

Fatty acyl-CoAs, like acetyl-CoA, are high energy compounds; their hydrolysis to free fatty acid and CoA has a large, negative standard free-energy change ($\Delta G^{10} \approx -31$ kJ/mol). The formation of a fatty acyl-CoA is made more favorable by the hydrolysis of two high-energy bonds in ATP; the pyrophosphate formed in the activation reaction is immediately hydrolyzed by inorganic pyrophosphatase, which pulls the preceding activation reaction in the direction of fatty acyl-CoA formation. The overall reaction is

\[
\text{Fatty acid} + \text{CoA} + \text{ATP} \rightarrow \text{Fatty acyl-CoA} + \text{AMP} + 2\text{Pi} \quad \Delta G^{10} = -34\text{KJ/mol}
\]

Fatty acyl-CoA esters found in the outer mitochondrial member do not cross the inner mitochondrial membrane intact. Instead, the fatty acyl group is transiently attached to the hydroxyl group of carnitine to form fatty acetyl carnitine. This trans-esterification is catalyzed by carnitine acyl-transferase I, on the outer face of the inner membrane. The fatty acyl-carnitine ester then enters the matrix by facilitated diffusion through the acyl-carnitine/carnitine transporter of the inner mitochondrial membrane.
Fig. 4. Fatty acid entry into mitochondria via the acyl-carnitine/carnitine transporter.

After fatty acyl-carnitine is formed at the outer surface of the inner mitochondrial membrane, it moves into the matrix by facilitated diffusion through the transporter. In the matrix, the acyl group is transferred to mitochondrial coenzyme A, freeing carnitine to return to the intermembrane space through the same transporter. The enzymes acyltransferase I and II are bound to the outer and inner surfaces, respectively, of the inner membrane. Aetyltransferase I is inhibited by malonyl-CoA, the first intermediate in fatty acid synthesis. This inhibition prevents the simultaneous synthesis and degradation of fatty acids.

In the third and final step of the entry process, the fatty acyl group is enzymatically transferred from carnitine to intra-mitochondrial coenzyme A by carnitine acyl transferase II. This isozyme, located on the inner face of the inner mitochondrial membrane, regenerates fatty acyl-CoA and releases it, along with free carnitine, into the matrix (fig. 4). Carnitine reenters the space between the inner and outer mitochondrial membranes via the acyl carnitine/carnitine transporter.

This three-step process for transferring fatty acids into the mitochondrial esterification to CoA, trans-esterification to carnitine followed by transport, and trans-esterification back to CoA-links two separate pools of coenzyme A, one in the cytosol, the other in mitochondria. These pools have different functions. Coenzyme A in the mitochondrial matrix is largely used in oxidative degradation of pyruvate, fatty acids, and some amino acids, whereas cytosolic enzyme A is used in the biosynthesis of fatty acid. The carnitine mediated entry process is the rate limiting step for oxidation of fatty acids in mitochondria. Once inside the mitochondrion, the fatty acyl-CoA is quickly acted upon by a set of enzymes in the matrix.

Suggested readings:

1. Introduction to plant physiology by Hopkins, W.G. Publisher- John Wiely & sons, Inc, New York, USA.

2. Plant physiology (4th edition) by Salisbury .F.B. and Rose .C.W. Publisher- Wadsworth publishing company, California, USA


4. Fundamentals of biochemistry by D.Voet Publisher- John Wiely & sons, Inc, New York, USA.

5. Principles of biochemistry by Leninger. Publisher: W. H. Freeman