CONTENTS

CHAPTER

21

Glycolysis

Fermentation

(*a*) **Sprinters at the end of a race.** A runner's respiratory and circulatory systems cannot supply oxygen to her leg muscles fast enough to keep up with the demand for energy, so glycolysis and lactate fermentation must provide the ATP. Panting after the race brings in the oxygen needed to remove the lactate through cellular respiration.
(*b*) **Bread.** Bread rises as CO_2 is liberated by fermenting yeast, which converts glucose to ethanol *via* the alcoholic fermentation pathway.

Carbohydrates are the first cellular constituents formed by photosynthetic organisms and result from the fixation of CO_2 on absorption of light. The carbohydrates are metabolized to yield a vast array of other organic compounds, many of which are subsequently utilized as dietary constituents by animals.The animals ingest great quantities of carbohydrates that can be either stored, or oxidized to obtain energy as ATP, or converted to lipids for more efficient energy storage or used for the synthesis of many cellular constituents.

The major function of carbohydrates in metabolism is as a fuel to be oxidized and provide energy for other metabolic processes. *The carbohydrate is utilized by cells mainly as glucose.* The 3 principal monosaccharides resulting from digestive processes are glucose, fructose and galactose. Much of the glucose is derived from starch which accounts for over half of the fuel in the diets of most humans. Glucose is also produced from other dietary components by the liver and, to a lesser extent, by the kidneys. *Fructose* results on large intake of sucrose while *galactose* is produced when lactose is the principal carbohydrate of the diet. Both fructose and galactose are easily converted to glucose by the liver. It is thus apparent that glucose is the major fuel of most organisms and that it can be quickly metabolized from glycogen stores when there arises a sudden need for energy.

Pentose sugars such as arabinose, ribose and xylose may be present in the diet. But their fate after absorption is, however, obscure.

GENERAL CONSIDERATIONS OF GLYCOLYSIS

Glycolysis (*glycos*[G] = sugar (sweet); *lysis*[G] = dissolution) is the sequence of 10 enzyme-catalyzed reactions that converts glucose into pyruvate with the simultaneous production of ATP. Moreover, glycolysis also includes the formation of lactate from pyruvate. The glycolytic sequence of reactions differs from one species to the other only in the mechanism of its regulation and in the subsequent metabolic fate of the pyruvate formed. In aerobic organisms, glycolysis is the prelude to the citric acid cycle and the electron transport chain which together harvest most of the energy contained in glucose. In fact, glycolysis is the central pathway of glucose catabolism.

Glycolysis takes place in the extramitochondrial part of the cell (or the soluble cytoplasm). It is frequently referred to as Embden-Meyerhof-Parnas or EMP pathway, in honour of these poineer workers in the field, and still represents one of the greatest achievements in the field of biochemistry. Other illustrious investigators, who contributed significantly to the final elucidation of glycolytic pathway, include Fritz A. Lipmann, Harden and Young, A.V. Hill, Carl Neuberg, Otto Warburg, and Carl F. Cori and his wife Gerty T. Cori.

Gustave Embden (LT,1874-1933)– A German biochemist, one of the great poineers of metabolic studies.
Otto Meyerhof (LT, 1883-1951)– Another German biochemist, a Nobel Laureate of 1992; sought refuge in the United States in 1938.
Jacob Parnas– Another leading biochemist on cell respiration.

There are 3 important routes taken by pyruvate after glycolysis, depending on the organism and the metabolic conditions (refer Fig. 21–1) :

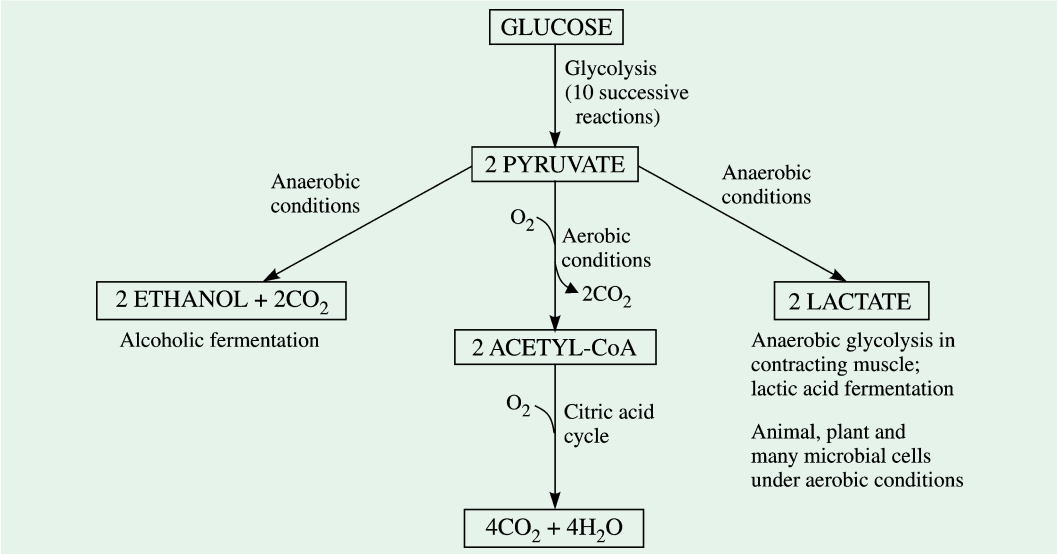


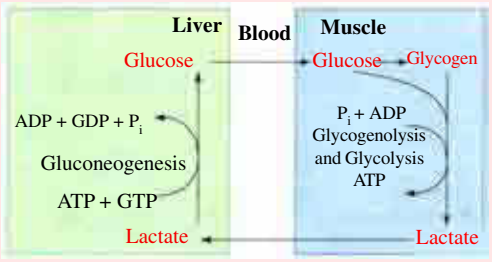
Fig. 21–1. Some important fates of glucose

(*a*) In aerobic organisms, the pyruvate so formed then enters mitochondria where it is oxidized, with the loss of its carboxyl group as CO_2, to form the acetyl group of acetyl-coenzyme A. Later, the acetyl group is completely oxidized to CO_2 and H_2O by the citric acid cycle with the intervention of molecular oxygen. This pathway is followed by aerobic animal and plant cells.

(*b*) If the supply of oxygen is insufficient, as in vigorously contracting skeletal muscles, the pyruvate cannot be oxidized further for lack of oxygen. Under such conditions, it is then reduced to lactate, a process called *anaerobic glycolysis*. Lactate is also produced from glucose in anaerobic microorganisms that carry out *lactic acid fermentation*.

CARL FERDINAND CORI AND GERTY THERESA CORI

Carl, a Czech-born American biochemist at Washington State University, shared the **1947 Nobel Prize for Medicine or Physiology** with his wife **Gerty** and **B.A. Houssay** from Argentina for their work on the metabolism of glycogen. They showed that the cells used an enzyme called *phosphorylase* to convert the stored glycogen to glucose, the sugar form which normally meets energy requirements of all cells in the body including muscle cells. They also found that the enzyme exists in either of its 2 forms: active or inactive. *The breakdown of glycogen to glucose is, in fact, a classic biochemical reaction that has bred three separate Nobel Prizes*. Both Carl and Gerty Cori discovered the Cori cycle. In essence, in the Cori cycle (adjoining figure) there is cycling of glucose due to glycolysis in muscle and gluconeogenesis in liver. In fact, lactate produced in muscles by glycolysis is transported by the blood to the liver. Gluconeogenesis in the liver converts the lactate back to glucose, which can be carried back to the muscles by the blood. Glucose can be stored in the muscles as glycogen until it is degraded by glycogenolysis.



(*c*) In some microorganisms (*e.g.*, brewer's yeast), the pyruvate formed from glucose is transformed anaerobically into ethanol and CO_2, a process called *alcoholic fermentation*. Since living organisms first arose in an atmosphere devoid of oxygen, anaerobic breakdown of glucose is the most ancient type of biological mechanism for obtaining energy from organic fuel molecules (Lehninger AL, 1984).

OTTO H. WARBURG (LT, 1883-1970) Warburg, a student of Emil Fischer, is considered by many to be the greatest biochemist of the first half of twentieth century. His first publication (with Fischer) appeared in 1904, and his last in 1970, the year of his death at age 87.

Two Phases of Glycolysis

During glycolysis, the 6-carbon glucose is broken down into two moles of 3-carbon pyruvate *via* 10 enzyme-catalyzed sequential reactions. These reactions are grouped under 2 phases, phase I and II (refer Figs. 21–2 and 21-3).

A. **Phase I** or **Preparatory Phase.** It consists of the first 5 steps. In these reactions, glucose is enzymatically phosphorylated by ATP (first at carbon 6 and later at carbon 1) to yield fructose 1,6-diphosphate which is then split in half to yield 2 moles of the 3-carbon compound, glyceraldehyde 3-phosphate. The first phase of glycolysis, thus, results in cleavage of the hexose chain. This phase requires an investment of 2ATP moles to activate (or *prime*) the glucose mole and prepare it for its cleavage into two 3-carbon pieces. Besides glucose, other hexoses such as D-fructose, D-galactose and D-mannose may also convert into glyceraldehyde 3-phosphate.

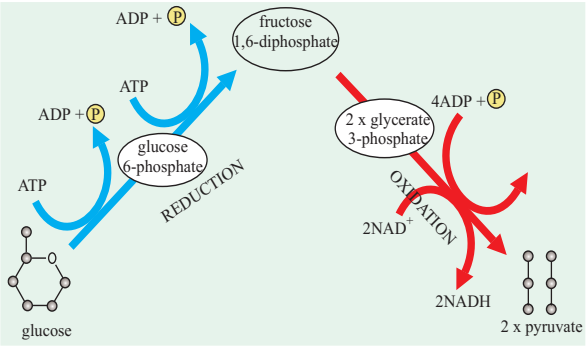


Fig 21-2. Glycolysis is a two-stage process

The 'uphill' part involves raising glucose to a higher energy level by using ATP. In the 'downhill' part, the products are oxidized, yielding 2 molecules of pyruvate, 2 molecules of reduced coenzyme and a net gain in ATP.

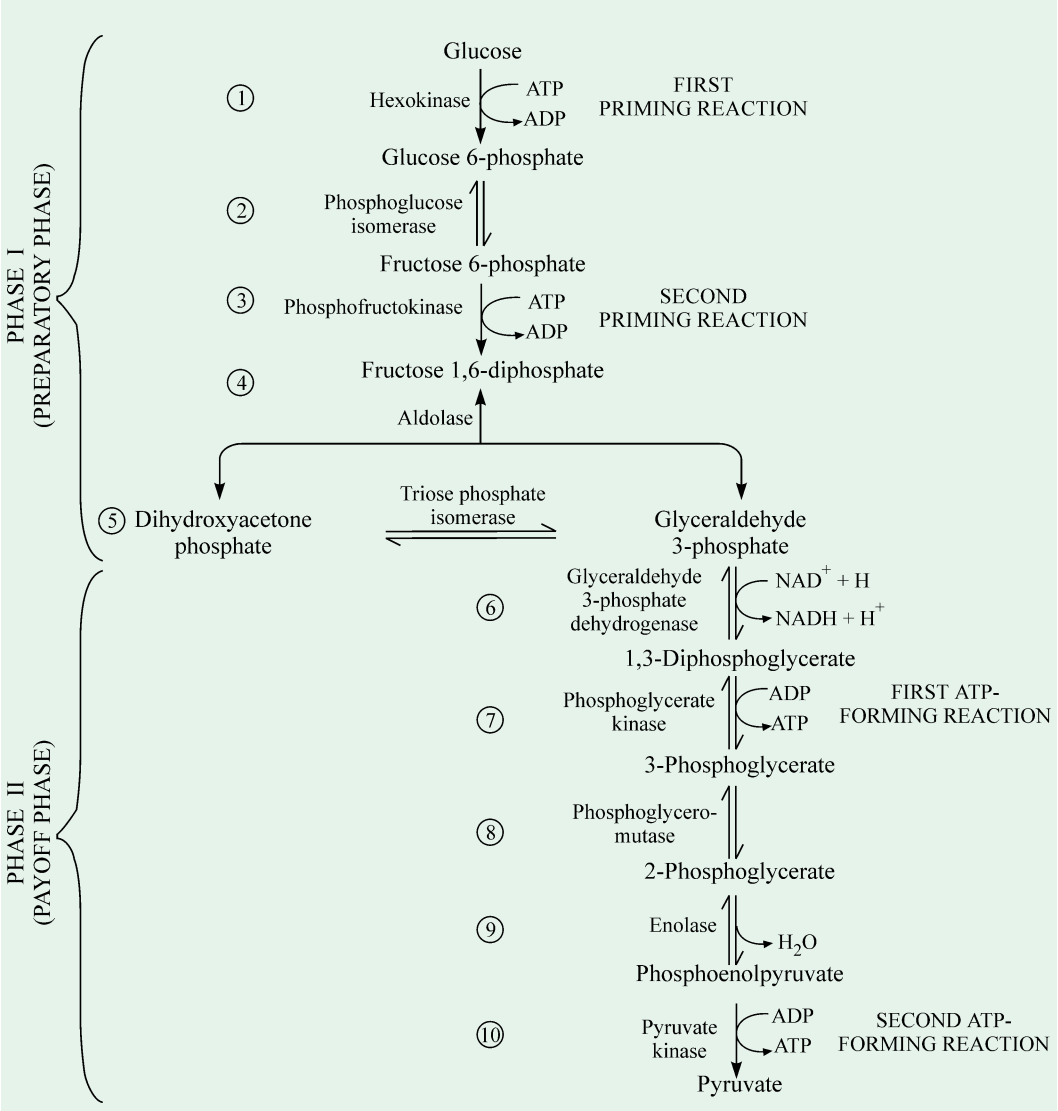


Fig. 21–3. Two phases of glycolysis

B. Phase II or **Payoff Phase.** The last 5 reactions of glycolysis constitute this phase. This phase represents the payoff of glycolysis, in which the energy liberated during conversion of 3 moles of glyceraldehyde 3-phosphate to 2 moles of pyruvate is converted by the coupled phosphorylation of 4 moles of ADP to ATP. Although 4 moles of ATP are formed in phase II, the net overall yield is only 2 moles of ATP per mole of glucsoe oxidized, since 2 moles of ATP are invested in phase I. The phase II is, thus, energy conserving.

A noticeable feature of glycolysis is that each of the 9 metabolic intermediates between glucose and pyruvate is a phosphorylated compound. The phosphoryl groups in these compounds are in either *ester* or *anhydride* likage. The phosphoryl or phosphate groups perform the following 3 functions :

1. The phosphate groups are completely ionized at *p*H 7, so that each of the 9 intermediates of glycolysis gains a net negative charge. Since cell membranes are, in general, impermeable to charged molecules, the glycolytic intermediates cannot escape from the cell. Only

glucose can enter cells and pyruvate or lactate can leave cells because cell membranes have specific transport systems that allow these molecules to pass.

2. The phosphate groups are essential components in the conservation of energy since they are ultimately transferred to ADP to produce ATP.
3. The phosphate groups act as recognition or binding groups required for the proper fit of the glycolytic intermediates to the active site of their corresponding enzymes.

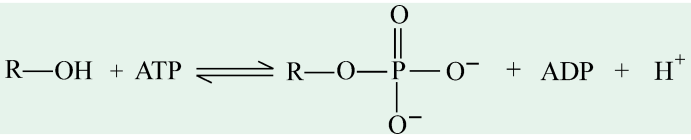
Enzymes Involved in Glycolysis

In most kinds of cells, the enzymes that catalyze glycolytic reactions (refer Table 21–1) are present in the extramitochondrial soluble fraction of the cell, the *cytosol*. On the other hand, the enzymes involved in citric acid cycle are located in the mitochondrial membrance in eukaryotes and in the plasma membrane in prokaryotes. *A remarkable feature of the glycolytic enzymes is that nearly all of them require* Mg^{2+} *for activity.*

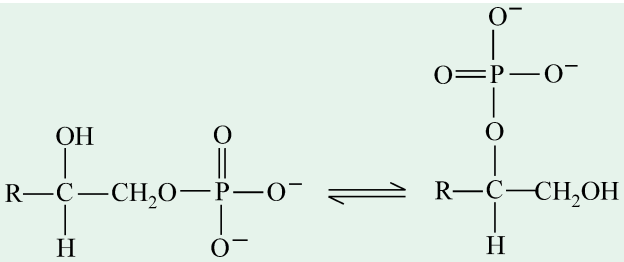
Kinds of Reactions in Glycolysis

For a better understanding of the various reactions of glycolysis, some of the kinds of reactions that occur in glycolysis are listed below :

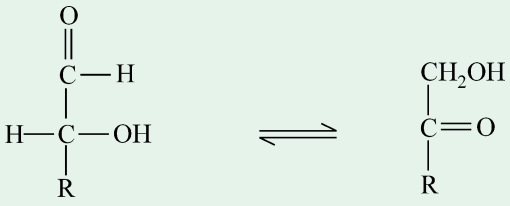
1. **Phosphoryl transfer.** A phosphate group is transferred from ATP to a glycolytic intermediate or *vice versa*.



2. **Phosphoryl shift.** A phosphate group is shifted within a molecule from one oxygen atom to another.



3. **Isomerization.** An aldose is converted into a ketose or *vice versa*.



4. **Dehydration.** A molecule of water is eliminated.

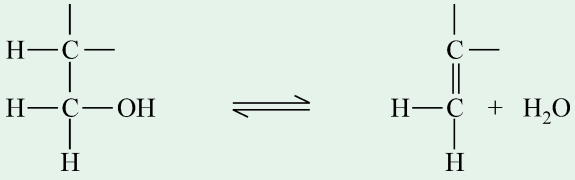


Table 21-1. Enzymes and reaction types of glycolysis

Step No.	*Enzyme*	*Enzyme Commission Number*	*Coenzyme (s) and Cofactor (s)*	*Activator (s)*	*Inhibitor (s)*	*Kind of Reaction Catalyzed*	*$\Delta G^{\circ\prime}$ kcal/mol*	*$\Delta G^{\circ *}$ kcal/mol*
1	Hexokinase	2.7.1.1	Mg^{2+}	ATP4–, Pi	Glucose 6-phosphate	Phosphoryl transfer	– 4.00	–8.0
2	Phosphoglucoisomerase	5.3.1.9	Mg2+	—	2-deoxyglucose 6-phosphate	Isomerization	+ 0.40	–0.6
3	Phosphofructokinase	2.7.1.11	Mg^{2+}	Fructose 2, 6-diphosphate, AMP, ADP, cAMP, K^{+}	ATP4–, citrate	Phosphoryl transfer	– 3.40	–5.3
4	Aldolase	4.1.2.7	Zn^{2+} (in microbes)	—	Chelating agents	Aldol cleavage	+ 5.73	–0.3
5	Phosphotriose isomerase	5.3.1.1	Mg^{2+}	—	—	Isomerization	+ 1.83	+ 0.6
6	Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	NAD	—	Iodoacetate	Phosphorylation coupled to oxidation	+ 1.50	+ 0.6
7	Phosphoglycerate kinase	2.7.2.3	M^{2+}	—	—	Phosphoryl transfer	– 4.50	+ 0.3
8	Phosphoglycerate mutase	5.4.2.1	Mg^{2+} 2, 3-diphos-phoglycerate	—	—	Phosphoryl shift	+ 1.06	+ 0.2
9	Enolase	4.2.1.11	Mg^{2+}, Mn^{2+} Zn^{2+}, Cd^{2+}	—	Fluoride + phosphate	Dehydration	+ 0.44	– 0.8
10	Pyruvate kinase	2.7.1.40	Kg^{2+}, K^{+}	—	Acetyl CoA, alanine, Ca^{2+}	Phosphoryl transfer	– 7.50	– 4.0

*ΔG, the actual free energy change, has been calculated from $\Delta G^{o\prime}$ and known concentrations of reactants under typical physiologic conditions. Glycolysis can proceed only if the ΔG values of all reactions are negative. The small positive ΔG values of 3 of the above reactions indicate that the concentrations of metabolites *in vivo* in cells undergoing glycolysis are not precisely known.

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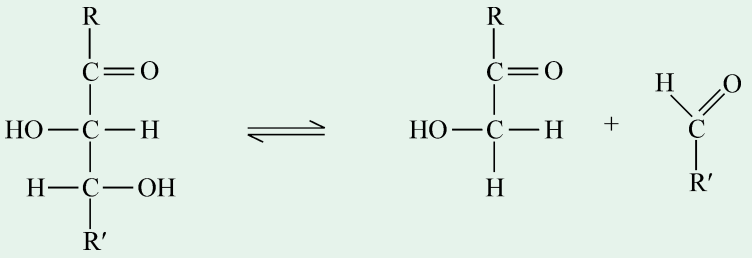
5. **Aldol cleavage.** A C–C bond is split in a reversal of an aldol condensation.



REACTION STEPS OF GLYCOLYSIS

The various reaction steps of glycolysis are schematically represented in Fig. 21–3. The details of these reactions and those of the enzymes, which catalyze them, are given below :

Step 1 : *Phosphorylation of Glucose*

In the first step, glucose is activated (or *primed*) for subsequent reactions by its phosphorylation at C_6 to yield glucose 6-phosphate, using ATP as phosphate donor. It is phosphoryl transfer type of reaction and is catalyzed by the inducible enzyme *hexokinase*, found in most animal, plant and microbial cells, and by an additional enzyme in the liver, *glucokinase*. The reaction is accompanied by considerable loss of free energy as heat. It is physiologically irreversible reaction because of the relatively low energy character of glucose 6-phosphate and the lower stability of Mg^{2+}.ADP compared to Mg^{2+}.ATP. Glucose 6-phosphate is an important compound, being at the junction of many metabolic pathways such as glycolysis, glycogenolysis, gluconeogenesis and the hexose monophosphate shunt.

The terms used for glyceric acid, pyruvic acid and lactic acid are respectively, glycerate, pyruvate and lactate. These terms are used to emphasize that at pH of the cell, the acid involved in the reaction is largely in dissociated form.

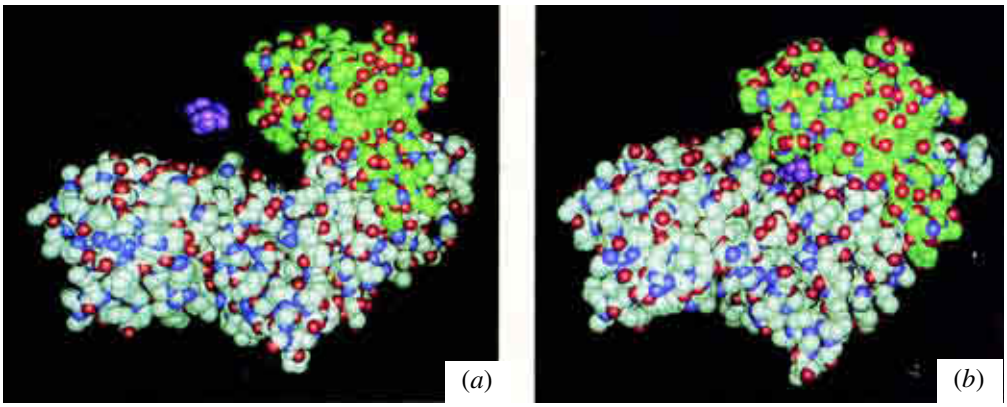
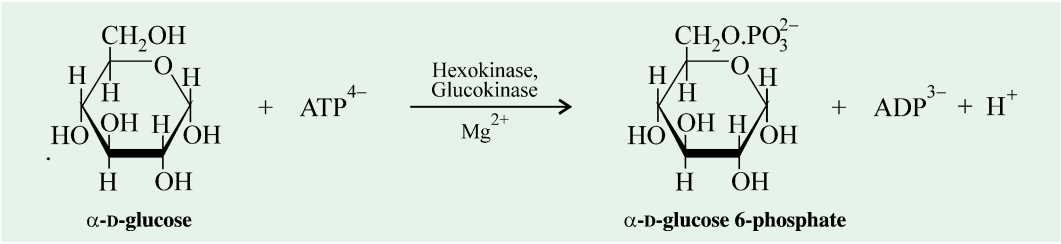


Fig. 21–4. A space-filling model of a subunit of yeast hexokinase in the "open" (a) and "closed" (b) conformations

The glucose, with which the enzyme complexes, is shown in purple. Note the prominant bilobal appearance of the free enzyme (the C atoms in the small lobe are shaded green, whereas those in the large lobe are light grey; the N and O atoms are blue and red). In the enzyme-substrate complex, these 2 lobes have swung together so as to engulf the substrate. Hexokinase also has been crystallized with a bound analogue of ATP. In the absence of glucose, the enzyme with the bound ATP analogue remains in the open conformation. The structural change caused by glucose results in the formation of additional contacts between the enzyme and ATP. This can explain why the binding of glucose enhances the binding of ATP.

(*Courtesy of Dr. Thomas A. Steiz, Yale University*)



Hexokinase (Fig. 21–4) is found in all tissues and exists in 3 isoenzyme forms, types I, II and III. Each form is composed of a single subunit (MW = 100,000). Brain contains chiefly type I and skeletal muscle, the type II. *All the 3 types are present in human liver and fat*. Type I is found in the cytosol or bound to the mitochondria, whereas type II exists primarily in the cytosol. Hexokinase, like all other kinases, requires Mg^{2+} (or other divalent metal ions such as Mn^{2+}, Ca^{2+} etc) for activity. The 2 lobes of hexokinase remain separate in the absence of its substrate molecule, *i.e.*, glucose. However, the conformation changes markedly on binding with glucose and the 2 lobes of the enzyme come together and surround the substrate. This induced fit is shown in Fig, 21–5. Hexokinase not only acts on glucose but also on some other common hexoses such as fructose and mannose. The activity of hexokinase is inhibited by the product of the raction (*i.e.*, glucose 6-phosphate) which binds the enzyme at an allosteric site. Hexokinase has a high affinity (*i.e.*, low Km value of about 1.0 mM) for its substrate, glucose. The reverse reaction (Glucose 6-phosphate → Glucose) requires a different enzyme, glucose 6-phosphatase with Mg^{2+} as cofactor. This reaction occurs in liver but not in muscle which lacks glucose 6-phosphatase. Yeast hexokinase, however, differs somewhat from the mammalian forms. It is a dimer of identical subunits (MW = 50,000). Also, its activity is not affected by glucose 6-phosphate.

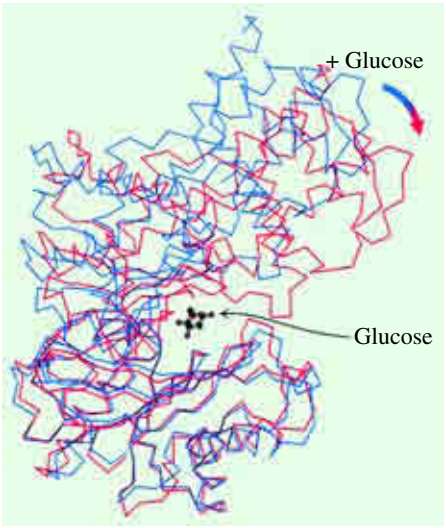


Fig. 21–5. Computer graphics of the induced fit in hexokinase
As shown in blue, the two lobes of hexokinase are separated in the absence of glucose. The conformation of hexokinase changes markedly on binding glucose, as shown in red. The two lobes of the enzyme come together and surround the substrate. Except around the binding site, the remaining portion of the enzyme in both the conditions (free and bound) is almost superimposed as seen in the figure.

Glucokinase (often designated **hexokinase IV**) is a monomeric inducible enzyme (MW = 48,000) and is found almost exclusively in the liver. Glucokinase differs from mammalian hexokinase in 3 respects :

(*a*) It is specific for glucose and does not act on other hexoses.

(*b*) It is not inhibited by glucose 6-phosphate.

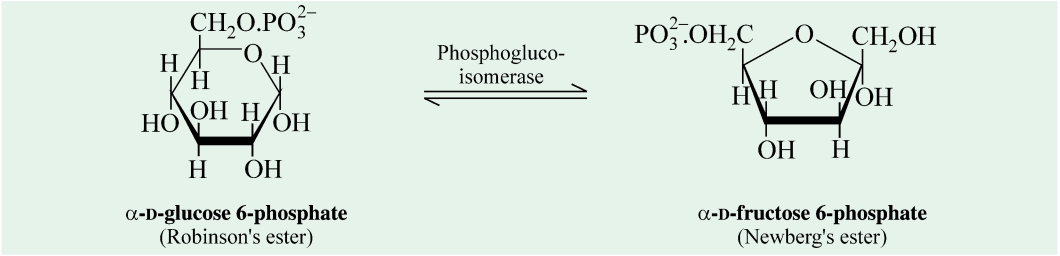
(*c*) It has a low affinity (*i.e.*, a much higher Km value of about 10 *m*M) for glucose than hexokinase.

The function of glucokinase is to remove glucose from the blood following a meal and to trap it in the liver cells, thereby allowing storage of glucose as glycogen or, after further metabolism, as fatty acids.

Step 2 : *Isomerization of Glucose 6-phosphate*

Glucose 6-phosphate is reversibly isomerized to frucose 6-phosphate by *phospho-glucoisomerase*. Thus, the 6-membered pyranose ring of glucose 6-phosphate is converted into the 5-membered furanose ring of fructose 6-phosphate. This reaction involves a shift in the carbonyl

oxygen from C_1 to C_2, thus converting an aldose into a ketose. At equilibrium, the ratio of aldose to ketose is 7 : 3, *i.e.*, glucose 6-phosphate predominates, having concentration over twice that of fructose 6-phosphate. The reaction proceeds readily in either direction because of relatively small standard free energy change. Fructose 6-phosphate has metabolic fates other than glycolysis.



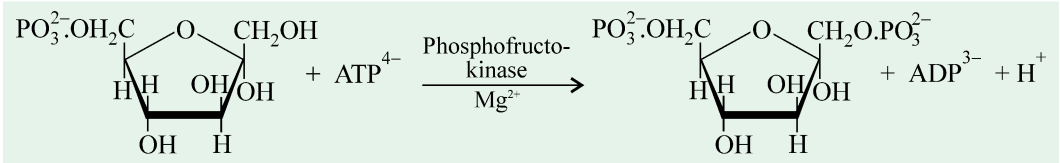
α-D-glucose 6-phosphate
(Robinson's ester)

α-D-fructose 6-phosphate
(Newberg's ester)

Human phosphoglucoisomerase (MW = 134,000) is a dimer of identical subunits and requires Mg^{2+} for activity. It is specific for glucose 6-phosphate and fructose 6-phosphate. An interesting sidelight of this enzyme is that it binds the α- pyranose form of glucose 6-phosphate, but the open chain form of fructose 6-phosphate. The α- and β-pyranose forms are interconvertible, so all of the glucose 6-phosphate is available to the enzyme.

Step 3 : *Phosphorylation of Fructose 6-phosphate*

This is the second of the two priming or activating reactions of glycolysis (the first one being Step 1). Fructose 6-phosphate is phosphorylated by ATP to produce fructose 1, 6-diphosphate in the presence of another inducible allosteric enzyme, *phosphofructokinase* (abbreviated as PFK). The enzyme catalyzes the transfer of a phosphate group from ATP to fructose 6-phosphate at C1 to yield fructose 1, 6-diphosphate. Since the reaction proceeds with $\Delta G^\circ = -3.4$ kcal/mol, it is essentially irreversible. *It is considered to be the committed step in glycolysis* since the PFK action 'commits' the cell to metabolizing glucose rather than storing or converting it to some other hexose. In addition to being a key step, it is an important control point of glycolysis.



D-fructose 6-phosphate
(Newberg's ester)

D-fructose 1, 6-diphosphate
(Harden-Young ester)

The muscle **phosphofructokinase** (MW = 320,000) *is one of the most complex known enzymes*. It is a tetramer of 4 identical subunits (Fig. 21–6)but dissociates into inactive dimers in the presence of citrate. Fructose 6-phosphate, however, promotes their reunion to form the tetramer. It is a major regulatory enzyme in muscle glycolysis because of its allosteric nature. The activity of phosphofructokinase is accelerated whenever the cell is deficient in ATP or there is an excess of ATP breakdown products (*i.e.*, ADP and AMP). The activity is, however, inhibited whenever the cell has plentiful ATP (and other fuels such as citrate or fatty acids) which lowers the affinity of the enzyme for fructose 6-phosphate. For the same reason, PFK is rightly regarded as the '*pace maker*' of glycolysis.

Step 4 : *Cleavage of Fructose 1,6-diphosphate*

This is a unique C–C bond scission reaction. Since fructose 1, 6-diphosphate is a molecule with phosphate group on both ends, it splits in the middle into two different triose phosphates, glyceraldehyde 3-phoshpate (an aldose) and dihydroxyacetone phosphate (a ketose). This reaction

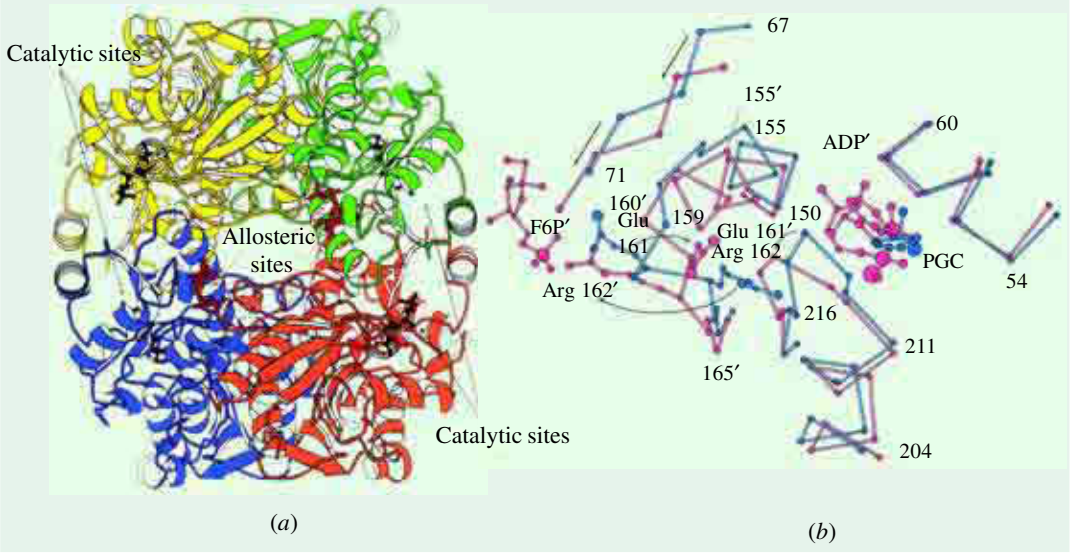


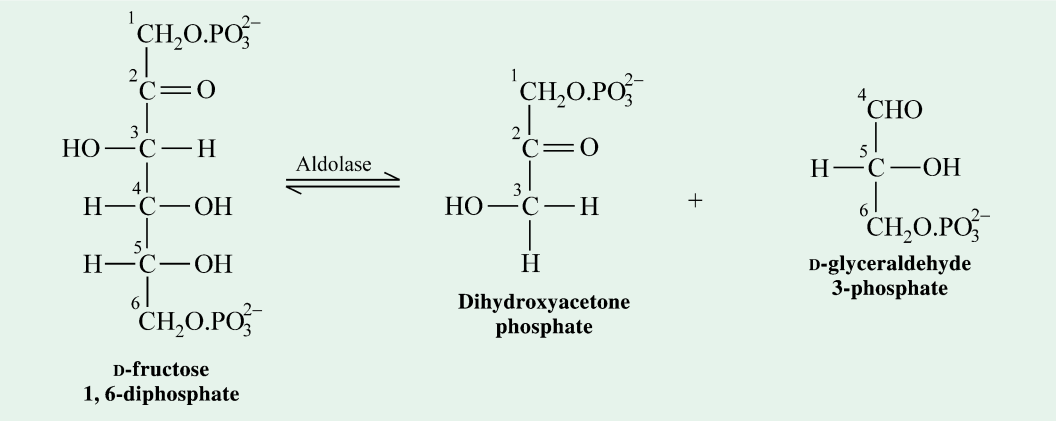
Fig. 21–6. The x-ray structure of phosphofructokinase (PFK)

(*a*) **Ribbon model.** Phosphofructokinase in the liver is a tetramer of four identical subunits. The positions of the catalytic and allosteric sites are indicated.

(*b*) **A superposition of those segments of the T state (*blue*) and R-state (*red*) enzymes that undergo a large conformational rearrangement upon the T→ R allosteric transition (*indicated by the arrows*).** Residues of the R state structure are marked by a prime. Also shown are bound ligands : the nonphysiological inhibitor 2-phosphoglycolate (PGC; a PEP analogue) for the T state, and the cooperative substrate F6P and the activator ADP for the R state.

(*Courtesy : (b) After Schirmer T and Evans PR, 1990*)

is catalyzed by the enzyme *fructose diphosphate aldolase* (often simply called *aldolase*) which cleaves the fructose 1, 6-diphosphate molecule between C_3 and C_4. Carbon atoms 4, 5 and 6 appear in glyceraldehyde 3-phosphate and 1, 2 and 3 in dihydroxyacetone phosphate. Although the aldolase reaction has a highly positive standard free energy change, it can readily proceed in either direction under the *p*H and concentration conditions existing in cells. Thus, this is a reversible aldol condensation type of reaction. The remaining steps in glycolysis involve 3 carbon units, rather than 6 carbon units.

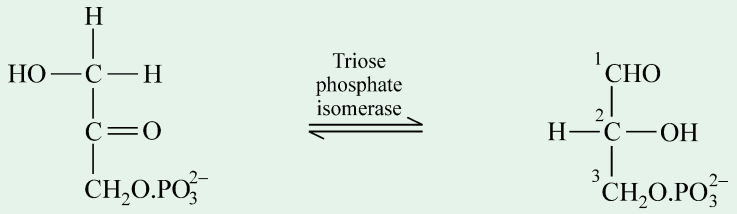


The **aldolase** (name derived from the nature of the reaction it catalyzes) from all animal tissues is a tetramer (MW = 160,000) but various tissues contain primarily one of the 3 different forms, characteristic of muscle, liver and brain respectively. All forms catalyze the above reaction

but at different rates. The aldolase of animal tissues does not require Mg^{2+} but in many microbes (yeast), aldolase needs Zn^{2+} for its activity.

Step 5: *Isomerization of Dihydroxyacetone phosphate*

Glyceraldehyde 3-phosphate (an aldose) can be directly degraded in the subsequent reaction steps of glycolysis but dihydroxyacetone phosphate (a ketose) cannot be. However, dihydroxyacetone phosphate can be readily and reversibly converted into glyceraldehyde 3-phosphate by the enzyme *triose phosphate isomerase* (also called *phosphotriose isomerase*) in the same way that glucose and fructose phosphates are interconverted by phosphoglucoisomerase. This is an isomerization reaction and occurs very rapidly. At equilibrium, about 95% of the triose phosphate is dihydroxyacetone phosphate. However, the reaction proceeds towards glyceraldehyde 3-phosphate formation because of the efficient removal of this product. It may be noted that by this reaction, carbon atoms 1, 2 and 3 of the starting glucose now become indistinguishable from carbon atoms 6, 5 and 4 respectively. This reaction completes the first phase of glycolysis.



Dihydroxyacetone phosphate (DHA-P) ⇌ **D-glyceraldehyde 3-phosphate (G 3-P)**

Triose phosphate isomerase (MW = 56,000) is a dimer of two identical subunits (Fig. 21– 7). It is noteworthy that the two enzymes, aldolase and triose phosphate isomerase have

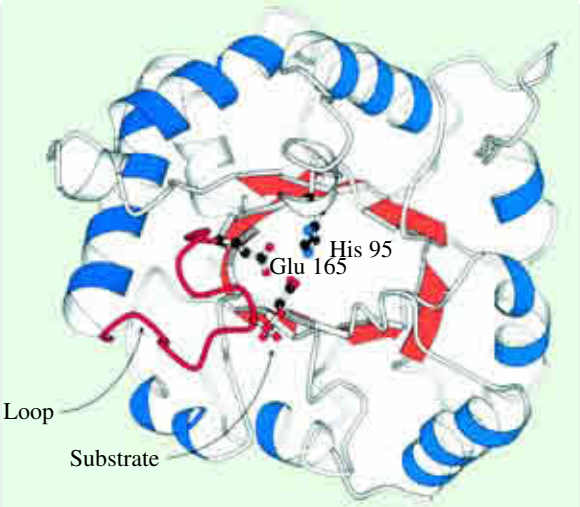


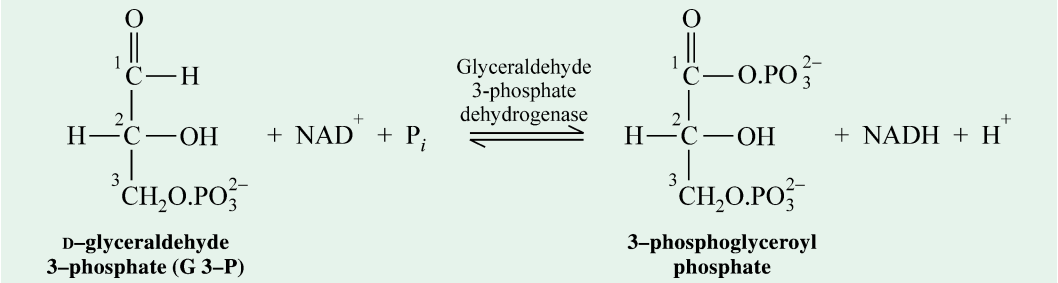
Fig. 21–7. Structure of triose phosphate isomerase

This enzyme consists of a central core of eight parallel β strands (orange) surrounded by eight α helices (blue). This structural motif, called an αβ barrel, is also found in the glycolytic enzymes, aldolase, enolase, and pyruvate kinase. Histidine 95 and glutamate 165, essential components of the active site of triose phosphate isomerase, are located in the barrel. A loop (red) closes off the active site on substrate binding.

a common substrate, dihydroxyacetone phosphate ; the difference being in their mode of action - the former produces fructose 1, 6-diphosphate whereas the latter, glyceraldehyde 3-phosphate.

Step 6 : *Oxidative Phosphorylation of Glyceraldehyde 3-phosphate*

This is first of the two energy-conserving reactions of glycolysis (the second one being Step 9) in which a high energy phosphate compound, 3-phosphoglyceroyl phosphate is formed. Here, glyceraldehyde 3-phosphate is converted into 3-phosphoglyceroyl phosphate (= 1, 3-diphosphoglycerate, 1,3-DPG) by the enzyme *glyceraldehyde* 3-phosphate *dehydrogenase* (= phosphoglyceraldehyde dehydrogenase), which is NAD^+-dependent. In this complex and reversible reaction, the aldehyde group of glyceraldehyde 3-phosphate is dehydrogenated, not to a free carboxyl group, but to a carboxylic anhydride with phosphoric acid, the 3-phosphoglyceroyl phosphate. This type of anhydride called an acyl phosphate has a very high standard free energy of hydrolysis ($\Delta G^{o\prime}$ = – 11.8 kcal/mol) and is thus a super high energy phosphate (refer Table 20–5). The acyl phosphate or 1,3–diphosphoglyceraldehyde conserves much of the free energy liberated during oxidation of the aldehyde group of glyceraldehyde 3-phosphate. The value of D$G^{o\prime}$ for this reaction is + 1.5 kcal/mol.



Glyceraldehyde 3-phosphate dehydrogenase (Fig. 21–8) from rabbit skeletal muscles (MW = 146,000) is a tetramer of 4 identical subunits, each consisting of a single polypeptide chain of about 330 amino acid residues. Four thiol (–SH) groups are present on each polypeptide, probably derived from cysteine residues within the polypeptide chain. One of the – SH groups is found at the active site on which an acyl-enzyme complex is formed. Each mole of enzyme also contains 4 moles of NAD^+. The enzyme is, however, inactivated by the – SH poison, iodoacetate which combines with the essential – SH group of the enzyme, thus preventing its participation in catalysis.

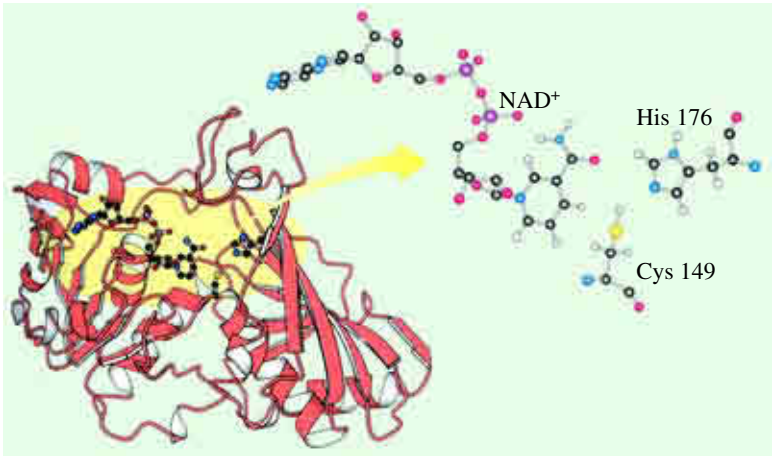


Fig. 21–8. Structure of glyceraldehyde 3-phosphate dehydrogenase
The active site includes a cysteine residue and a histidine residue adjacent to a bound NAD^+.

$$\underset{\text{Active enzyme}}{\text{Enzyme} - \text{SH}} + \underset{\text{Iodoacetate}}{\text{ICH}_2\text{COO}} \longrightarrow \underset{\text{Inactive enzyme}}{\text{Enzyme} - \text{S} - \text{CH}_2\text{COO}^-} + \text{HI}$$

The mechanism of action of glyceraldehyde 3-phosphate dehydrogenase is rather complex and resembles that of a-ketoglutarate dehydrogenase. It involves 3 steps (refer Fig 21–9).

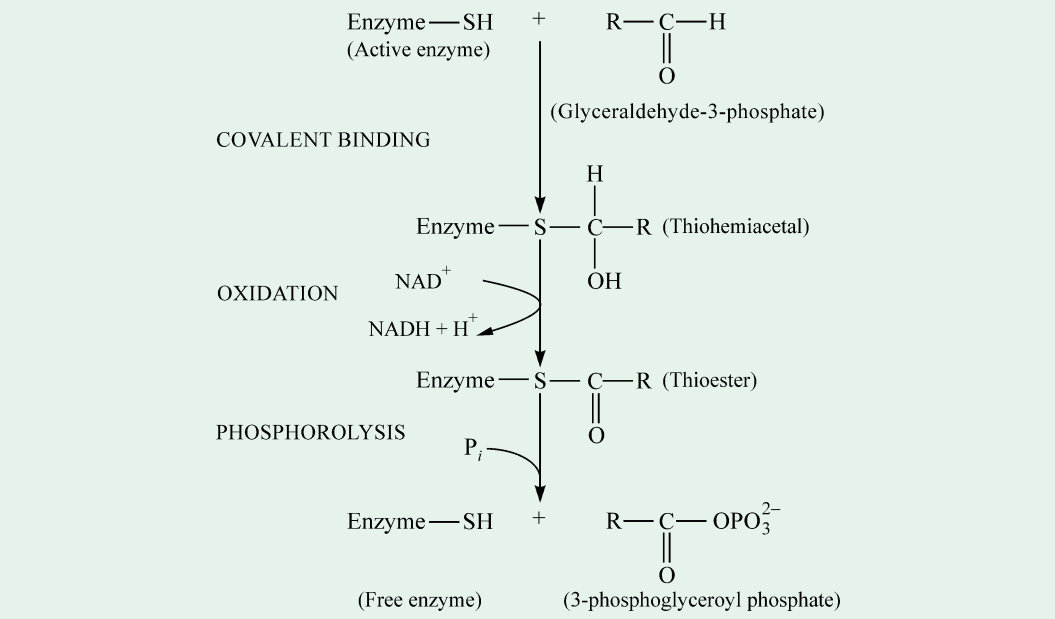
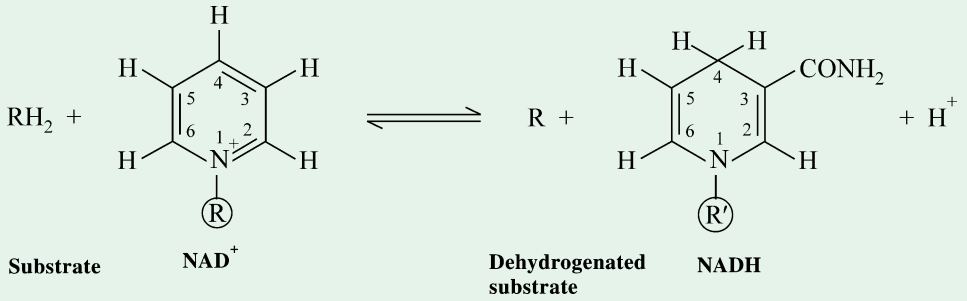


Fig. 21–9. Mechanism of action of glyceraldehyde 3-phosphate dehydrogenase, represented here as Enzyme—SH

(*a*) *Covalent binding of substrate to —SH group*. The substrate first combines with an —SH group of an essential cysteine residue present at the active site of the enzyme, forming a thiohemiacetal.

(*b*) *Oxidation of thiohemiacetal and reduction of NAD*$^+$. Thiohemiacetal (= hemithioacetal) is then oxidized to produce a high energy covalent acyl-enzyme complex, called thiol ester (= thioester). The hydrogen removed in this oxidation is transferred to the coenzyme NAD^+, also tightly bound at the active site of the enzyme molecule. The reduction of NAD^+ proceeds by the enzymatic transfer of a hydride ion ($:H^-$) from the aldehyde group of the substrate, glyceraldehyde 3-phosphate to position 4 of the nicotinamide ring of NAD^+, resulting in its reduction at ring positions 1 and 4 to yield the reduced coenzyme NADH. The other hydrogen atom of the substrate molecule appears in the medium as H^+. For the same reason, the enzymatic reduction of NAD^+ is written to include the hydrogen ion (H^+) formed. NADH formed in the reaction is reoxidized to NAD^+ in order to participate in the breakdown of more glucose molecules to pyruvate.

Hydride ion is a hydrogen nucleus and two electrons.



(*c*) *Phosphorolysis of thioester.* Finally, the acyl-enzyme (or thioester) reacts with an inorganic phosphate (Pi) forming an acyl phosphate called 3-phosphoglyceroyl phosphate and the free enzyme

with a reconstituted —SH group is liberated. A diagrammatic representation of the mechanism of action of glyceraldehyde 3-phosphate dehydrogenase is given in Fig. 21-10.

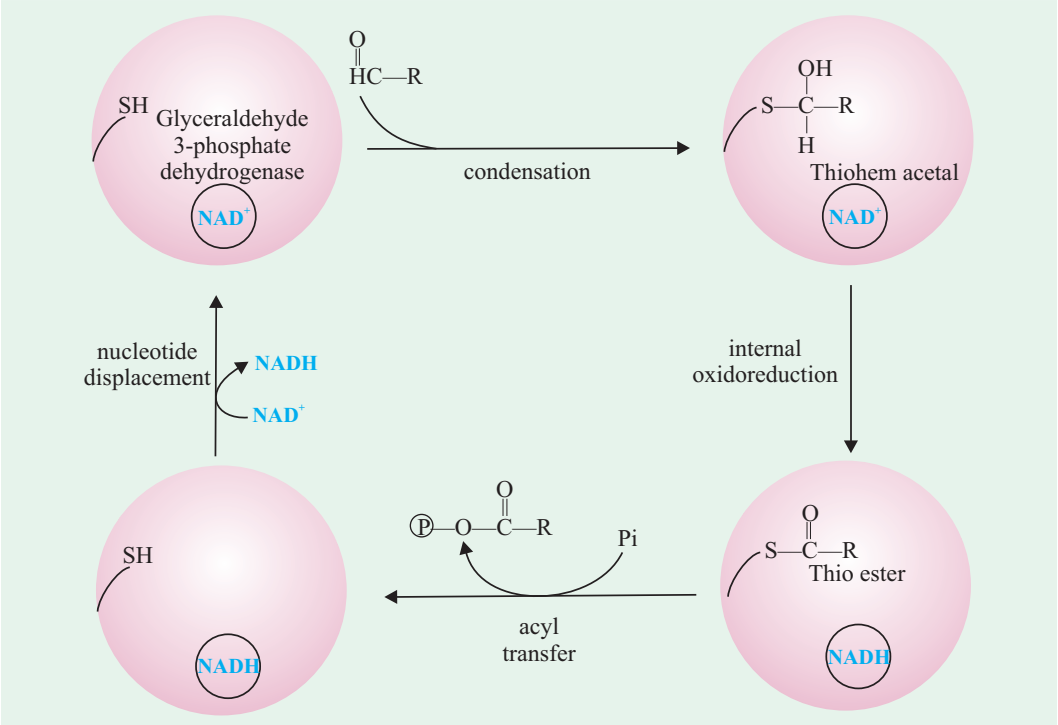
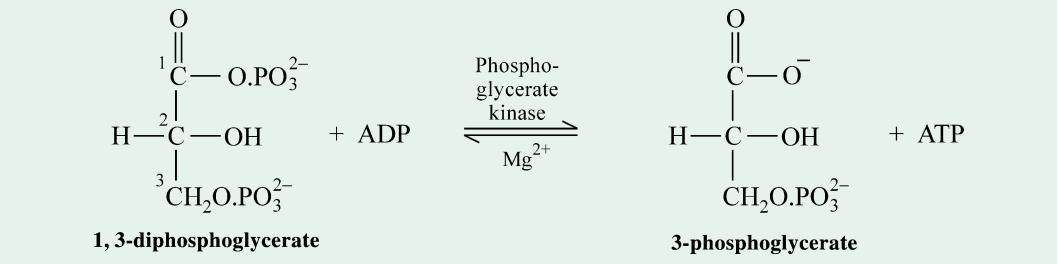


Fig. 21–10. Mechanism of action of glyceraldehyde 3-phosphate dehydrogenase

Large circle represents enzyme, small circle binding site for NAD^+; RCOH, glyceraldehyde 3-phosphate; — SH, the sulfhydryl group of the cysteine residue located at the active site ; and ~P, the high-energy phosphate bond of 1, 3-bisphosphoglycerate.

Step 7 : *Transfer of Phosphate from 1,3-DPG to ADP*

This is the first ATP-generating reaction in glycolysis (the second one being Step 10). It involves the transfer of high-energy phosphate group from the carboxylic group of 3-phosphoglyceroyl phosphate (= 1, 3-diphosphoglycerate or 1,3-DPG) to ADP by the enzyme phosphoglycerate kinase, thus producing ATP and leaving 3-phosphoglycerate. Since 2 moles of triose phosphate are produced per mole of glucose, 2 moles of ATP are generated at this stage per mole of glucose oxidized. The value of $\Delta G^{\circ\prime}$ for this essentially reversible reaction is – 4.5 kcal/mol.



Phosphoglycerate kinase (Fig. 21–11) has a molecular weight of about 45,000. As with other enzymes of this type, there is an absolute requirement for a divalent metal cofactor (Mg^{2+}, Mn^{2+}

or Ca^{2+}). The metal interacts with the ADP or ATP to form the reactive complex. This and the preceding reaction (*i.e.*, Steps 6 and 7) together constitute an energy-coupling process. The sum of these two sequential reactions would give :

Glyceraldehyde 3-phosphate + NAD^+ + Pi + ADP

$\rightleftharpoons$ 3-phosphoglycerate + ATP + NADH + H^+

$\Delta G^{\circ\prime}$ = (+1.5) + (– 4.5)

= – 3.0 kcal/mol

Thus, the net outcome of Steps 6 and 7 is :

(*a*) Glyceraldehyde 3-phosphate, an aldehyde, is oxidized to 3-phosphoglycerate, a carboxylic acid.

(*b*) NAD+ is reduced to NADH.

(*c*) ATP is produced from ADP and Pi.

In other words, the net result of Steps 6 and 7 is that the energy released on oxidation (or dehydrogenation) of an aldehyde to a carboxylate group is conserved by the coupled formation of ATP from ADP and Pi. Such reactions are called *substrate-level phosphorylations* because the energy required to bring them about arises from the dehydrogenation of an organic substrate molecule (for example, glyceraldehyde 3-phosphate in the present case). This term distinguishes these phosphorylations from *oxidative phosphorylation*, which is the formation of ATP coupled to the oxidation of NADH and $FADH_2$ by oxygen. As the oxidative phosphorylation is coupled to electron transport, it is also called as *respiratory-chain phosphorylation*.

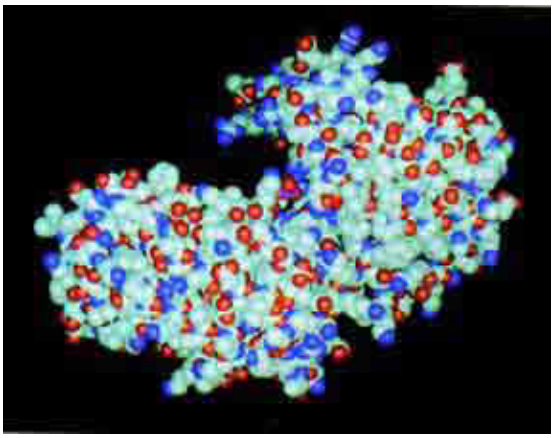


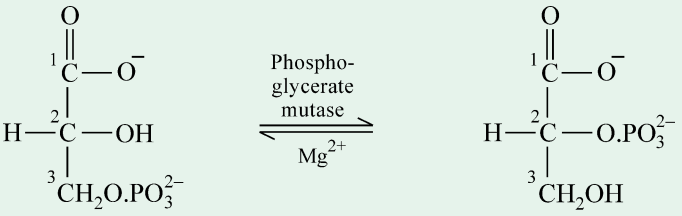
Fig. 21–11. A space-filling model of yeast phosphoglycerate kinase showing its deeply clefted bilobal structure

The substrate-binding site is at the bottom of the cleft as marked by the P atom (*purple*) of 3PG. Compare this structure with that of hexokinase (Fig. 21–4).

(*Courtesy of Herman Watson, University of Bristol, U.K.*)

Step 8 : *Isomerization of 3-phosphoglycerate*

The 3-phosphoglycerate is converted into 2-phosphoglycerate due to the intramolecular shift of phosphoryl group from C_3 to C_2, by the enzyme *phosphoglycerate mutase* (= *phosphoglyceromutase*). This is a reversible reaction with a $\Delta G^{\circ\prime}$ value = + 1.06 kcal/mol.



3-phosphoglycerate **2-phosphoglycerate**

Phosphoglycerate mutase (MW = 65,000) is a dimer of identical subunits. Mg^{2+} is essential for this reaction. The enzyme requires 2, 3-diphosphoglycerate as cofactor for its action and combines with it to give a phosphoenzyme and either the 2-phosphoglycerate or the 3-phosphoglycerate :

Enzyme—OH + 2, 3-diphosphoglycerate

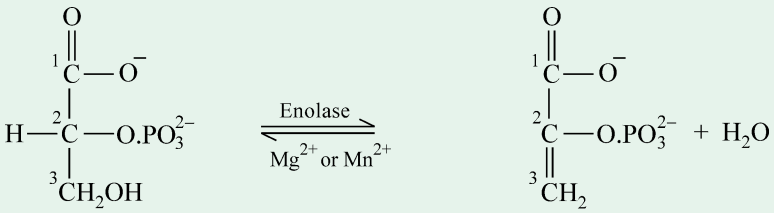
$\rightleftharpoons$ Enzyme — O.PO + 2- or 3-phosphoglycerate

At high concentration of 3-phosphoglycerate, the 3-phosphoglycerate and phosphoenzyme react to produce free enzyme and 2, 3-diphosphoglycerate, which in turn yields the 2-phosphoglycerate. The reaction occurs in reverse direction at high concentration of 2-phosphoglycerate. At equilibrium, the ratio of 2- to the 3-phosphate is 50 : 1. The 2, 3-diphosphoglycerate is produced from 3-phosphoglycerate by the enzyme *2, 3-diphosphoglycerate kinase* as follows :

3-phosphoglycerate + ATP ⟶ 2, 3 diphosphoglycerate + ADP

Step 9 : *Dehydration of 2-phosphoglycerate*

This is the second reaction of glycolysis in which is a high-energy phosphate compound (*i.e.*, phosphoenolpyruvate) is formed. The 2-phosphoglycerate is dehydrated by the action of enolase (= *phosphopyruvate hydratase*) to phosphoenolypyruvate (abbreviated as PEP), which is the phosphate ester of enol tautomer of pyruvate. This is a reversible reaction and has a relatively small free energy change value of + 0.44 kcal/mol. At equilibrium, the ratio of 2-phosphoglycerate to phosphoenolpyruvate is 2 : 1. However, phosphoenolpyruvate, and not 2-phosphoglycerate, contains a super high-energy phosphate bond. The loss of water from 2-phosphoglycerate causes a redistribution of energy within the molecule, raising the phosphate on position 2 to the high energy state, thus forming PEP. The reaction is freely reversible since there is little free energy change. The $\Delta G^{\circ\prime}$ value for the hydrolysis of PEP is –14.8 kcal/mol. There is more than sufficient energy to allow synthesis of ATP from PEP in the next step of glycolysis.

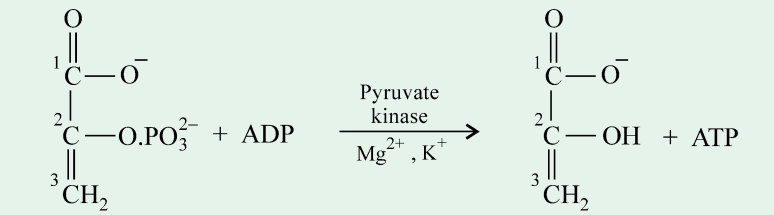


2-phosphoglycerate **Phosphoenolpyruvate**

Enolase (MW = 88,000) is a dimer with identical subunits. It requires Mg^{2+}, Mn^{2+}, Zn^{2+} or Cd^{2+} as cofactor in its active site which forms a complex with the enzyme before the substrate is bound. Enolase is inhibited by the simultaneous presence of fluoride and phosphate. In fact, the fluorophosphate ion, which binds with Mg^{2+} forming magnesium fluorophosphate, is the true inhibitor.

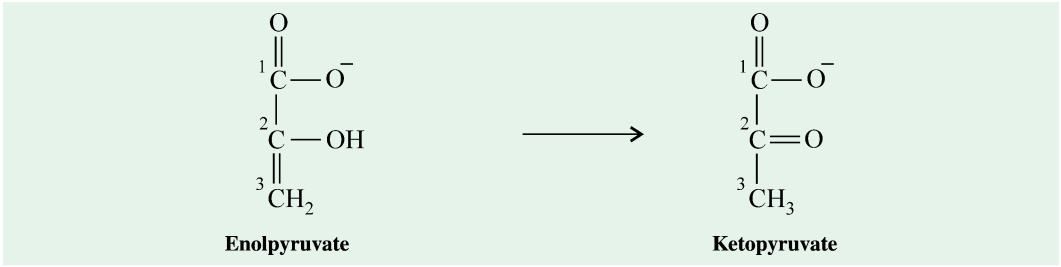
Step 10 : *Transfer of Phosphate from PEP to ADP*

This is the second ATP-generating reaction in glycolysis. Here, phosphoenolpyruvate (PEP) is converted into pyruvate in enol form (*i.e.*, enolpyruvate) by the inducible allosteric enzyme *pyruvate kinase* (abbreviated as *PK*). The enzyme catalyzes the transfer of a phosphoryl group from PEP to ADP, thus forming ATP. *This phosphorylation reaction is nonoxidative* in contrast with the one catalyzed by glyceraldehyde 3-phosphate dehydrogenase (*i.e.*, Step 6). This is another physiologically irreversible step in glycolysis (the first one being Step 1) and proceeds with $\Delta G^{\circ\prime}$ = – 7.5 kcal/mol.



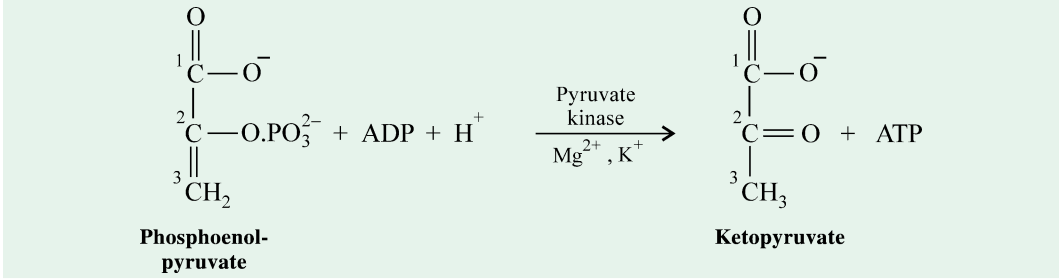
Phosphoenolpyruvate **Enolpyruvate**

The enolpyruvate, however, rearranges rapidly and *nonenzymatically* to yield the keto form of pyurvate (*i.e.*, ketopyruvate). The keto form predominates at *p*H 7.0.



Enolpyruvate **Ketopyruvate**

The point of equilibrium of this nonenzymatic reaction is very far to the right. Therefore, it 'drives' the preceding enzymatic reaction to the right by mass action. The two reactions, on addition, give :



Phosphoenol-pyruvate **Ketopyruvate**

The overall reaction has a very large negative $\Delta G^{\circ\prime}$ value due to the spontaneous conversion of enol form of pyruvate to the keto form. The $\Delta G^{\circ\prime}$ value for hydrolysis of PEP is – 14.8 kcal/mol. About half of this energy is recovered as ATP ($\Delta G^{\circ\prime}$ = – 7.3 kcal/mol) and the rest (–7.5 kcal/mol) constitutes a large driving force, pushing the reaction far to the right. Since 2 moles of PEP are formed per mole of glucose oxidized, 2 moles of ATP are also produced per mole of glucose. The conversion of phosphoenolpyruvate into pyruvate is the second example of *substrate-level phosphorylation* in glycolysis.

Pyruvate kinase (MW = 190,000 – 250,000) is found in 3 major forms : muscle and brain contain the M type and liver the L type whereas most other tissues contain the A type. Each type controls different catalytic properties in accordance with its differing roles it performs. However, all forms of the enzyme are tetramers of 4 identical subunits. The enzyme is dependent on the concentration of K^+ which increases the affinity of PEP for enzyme. The enzyme also requires Mg^{2+} as it forms a complex with ATP, Mg^{2+}. ATP complex, which is the actual substrate. The participation of both K^+ and Mg^{2+} ions is an unusual, although not unique, instance of complementary requirement for two different cations. Pyruvate kinase is an allosteric enzyme and like phosphofructokinase, its activity is regulated by several means. In general, its activity is high when a net flux of glucose to pyruvate or lactate is required and low during gluconeogenesis (*i.e.*, during formation of glucose or glycogen from noncarbohydrate sources). Pyruvate kinase is also inhibited by acyl-CoA and by long-chain fatty acids, both important fuels for citric acid cycle. Certain amino acids also modulate enzyme activity, *esp.*, in the liver.

In metabolic pathways, the enzymes catalyzing essentially irreversible reactions are the key sites of control. In glycolysis, the steps 1, 3 and 10 which are catalyzed by hexokinase (or glucokinase), phosphofructokinase and pyruvate kinase respectively are virtually irreversible. Hence, they perform regulatory as well as catalytic functions.

STOICHIOMETRY OF GLYCOLYSIS

Overall Balance Sheet

The word stoichiometry is pronounced as **'stoy-ke-om'–etry'.** It combines two Greek words : *stoicheion* = element and *metron* = measure.

Keeping in mind that each molecule of glucose yields 2 molecules of glyceraldehyde 3-phosphate, the total inputs and the outputs of all the 10 glycolytic reactions may be written as follows :

$$\text{Glucose} + 2\ \text{ATP} + 2\ \text{Pi} + 2\ \text{NAD}^+ + 2\ \text{H}^+ + 4\ \text{ADP} \longrightarrow 2\ \text{pyruvate} + 2\ \text{H}^+ + 4\ \text{ATP} + 2\ \text{H}_2\text{O} + 2\ \text{NADH} + 2\ \text{H}^+ + 2\text{ADP}$$

On cancelling the common terms, we get the net equation for the transformation of glucose into pyruvate :

$$\text{Glucose} + 2\ \text{Pi} + 2\ \text{ADP} + 2\ \text{NAD}^+ \longrightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}^+ + 2\ \text{H}_2\text{O}$$

Thus, three things happen simultaneously in glycolysis :

(*a*) Glucose is oxidized to pyruvate.

(*b*) NAD+ is reduced to NADH.

(*c*) ADP is phosphorylated to form ATP.

There can be no EMP pathway without all 3 events which means that NAD^+, ADP and Pi, as well as glucose, must be present.

Energy Yield

Further, 2 moles of ATP are generated in glycolysis. A summary of the steps in which ATP is consumed or formed is given in Table 21–2.

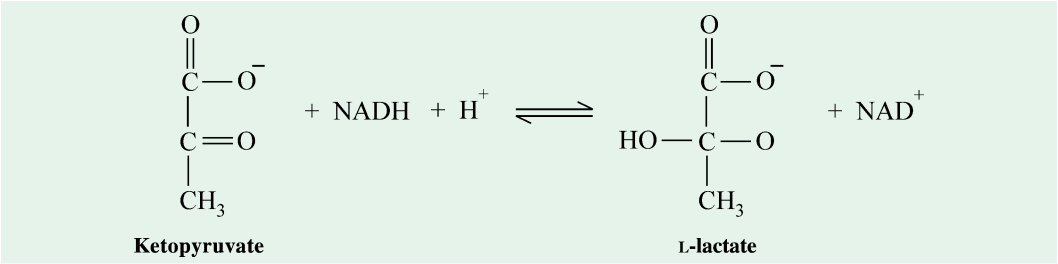
Table 21–2. Energy yield of glycolysis

Step	*Reaction*	*Consumption of ATP*	*Gain of ATP*
1	Glucose ⟶ Glucose 6-phosphate	1	
3	Fructose 6-phosphate ⟶ Fructose 1, 6-diphosphate	1	
7	1, 3-diphosphoglycerate ⟶ 3-phosphoglycerate		1 × 2 = 2
10	Phosphoenolpyruvate ⟶ Pyruvate		1 × 2 = 2
		2	4
		Net gain of ATP = 4 – 2 = 2	

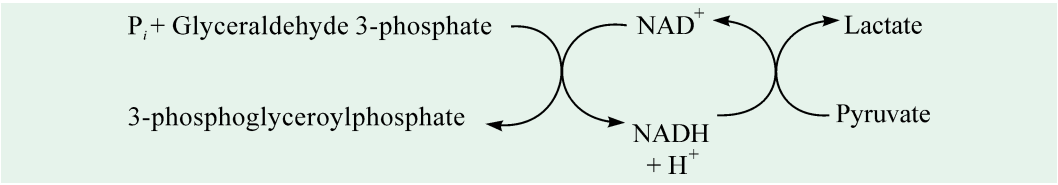
MUSCLE (OR ANAEROBIC) GLYCOLYSIS AND HOMOLACTIC FERMENTATION

In plant and animal tissues, under aerobic conditions, the pyruvate is the product of glycolysis and NADH, formed by the dehydrogenation of glyceraldehyde 3-phosphate, is then reoxidized to NAD^+ by oxygen. However, under anaerobic conditions, in actively contracting skeletal muscles, the NADH generated in glycolysis cannot be reoxidized by oxygen but must be reoxidized to NAD^+ by the pyruvate itself, converting pyruvate into lactate. Such type of glycolytic sequence occurring under anaerobic conditions in the muscle tissues is commonly spoken of as *muscle glycolysis or anaerobic glycolysis*. Besides the skeletal muscles, a large number of microorganisms, the lactic acid bacteria (esp., species of *Lactobacilli, Bacilli, Streptococci and Clostridia*) also follow the same path for the reduction of pyruvate to lactate. Such type of fermentation that yields lactate as the sole product is termed *homolactic fermentation.*

The reduction of pyruvate by NADH to form lactate is catalyzed by lactate dehydrogenase (abbreviated as LDH) which forms the L-isomer of lactate. As the reaction has a large negative value of



$\Delta G^{\circ\prime}$ (–6.0 kcal/mol), it proceeds far to the right. The reoxidation of NADH via lactate formation allows glycolysis to proceed in the absence of O_2 by regenerating sufficient NAD^+ for the reaction Step 6 of glycolysis, catalyzed by glyceraldehyde 3-phosphate dehydrogenase. Thus, the reduction of pyruvate to lactate is coupled to the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglyceroyl phosphate as shown below :



The anaerobic glycolysis, thus, results in the accumulation of 2 moles of lactate per mole of glucose utilized. *Lactate is one of the blind alleys or "dead-end streets" in metabolism and, once formed, it can only be reconverted to pyruvate.* The reconversion is, however, accomplished in the liver cells into which lactate is transported by circulation from muscle cells.

Lactate dehydrogenase (MW = 140,000) is a tetramer consisting of 4 subunits which are of 2 types. These are designated as M (for skeletal muscle) and H (for heart muscle) and differ in sequence. The skeletal muscle contains LDH of M_4 type mainly whereas heart muscle LDH is largely H_4. However, various tissues contain all possible hybrids, M_1H_3, M_2H_2 and M_3H_1. The exact amount of each of these 5 isozyme forms, as they are called, differs with the tissue. The various isozyme forms differ with respect to their K_m value for pyruvate, their turnover numbers or V_{max} and the degree of their allosteric inhibition by pyruvate. The H_4 has a low K_m for pyruvate and is strongly inhibited by pyruvate. It is, thus, better adapted for a more highly aerobic organ which removes lactate and oxidizes it to pyruvate mainly in the mitochondria. In contrast, the M_4 has a higher K_m for pyruvate and is not inhibited by pyruvate. Hence, it is useful in a tissue in which a more anaerobic environment may predominate as in skeletal muscle. *M_4 is catalytically more active than H_4.* Lactate dehydrogenase is characteristically inhibited by oxamate.

The net equation for anaerobic glycolysis in muscles and lactate fermentation in some microbes would then be :

$$\text{Glucose} + 2\ \text{Pi} + 2\ \text{ADP} \longrightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ \text{H+} + 2\ H_2O$$

No oxygen is consumed in anaerobic glycolysis. Two steps involve oxidation-reduction, the oxidation of glyceraldehyde 3-phosphate and the reduction of pyruvate to lactate. NAD^+ participates in both reactions. Hence, the two cancel out and there is no net oxidation or reduction.

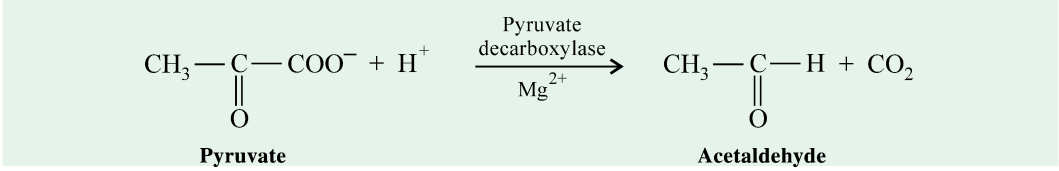
In anaerobic glycolysis, however, there occurs no net change in the oxidation state of carbon. This becomes evident by comparing the empirical formula of glucose ($C_6H_{12}O_6$) with that of lactic acid ($C_3H_6O_3$). The ratio of C to H to O is the same (*i.e.*, 1 : 2 : 1) for both, showing that no net oxidation of carbon has occurred. Nevertheless, some of the energy of glucose is extracted by

anaerobic glycolysis, sufficient to give a net gain of 2 ATP per glucose utilized. This is equivalent to an estimated 15 kcal. Since some 56 kcal per mole are produced when glucose is degraded to lactate under standard conditions, the overall efficiency of glycolysis is 15/56 × 100 or approximately 25% – a rather high figure !

ALCOHOLIC FERMENTATION

In yeast and other microorganisms, the reactions of glycolysis up to pyruvate formation are identical to those described for anaerobic glycolysis and the difference occurs only in its terminal steps. In contrast to animals, which utilize lactate dehydrogenase reaction for the reoxidation of NADH to generate NAD^+, the yeast cells utilize two enzymatic reactions for the purpose, as lactate dehydrogenase is not found in them :

(*a*) In the first step, the pyruvate resulting from glucose breakdown is decarboxylated by the action of *pyruvate decarboxylase* (= *2, oxo-acid carboxylase*) to produce acetaldehyde and carbon dioxide. This is an irreversible reaction and does not involve the net oxidation of pyruvate.

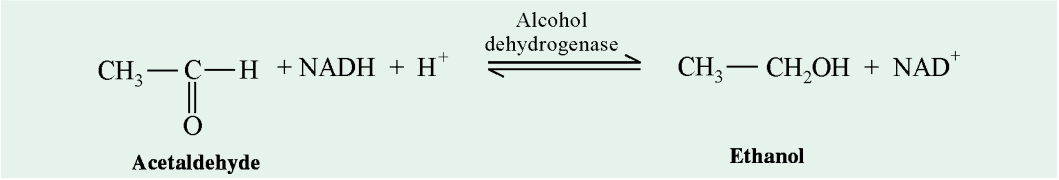


Pyruvate decarboxylase (E.C. 4.1.1.1) requires the usual Mg^{2+} for activity although certain trivalent ions such as Al^{3+}, Fe^{3+} may also satisfy the need. It has a tightly bound coenzyme, thiamine pyrophosphate, TPP (= cocarboxylase). TPP functions as a transient carrier of acetaldehyde group. In fact, the carboxyl group of pyruvate is lost as CO_2 and the rest of the molecule (sometimes referred to as *active acetaldehyde*) is simultaneouly transferred to the position 2 of the thiazole ring of TPP, to yield its hydroxyethyl derivative. This is unstable since the hydroxyethyl group quickly dissociates from the coenzyme to yield free acetaldehyde.

$$\text{Pyruvate} + H_2O + \text{TPP-E} \longrightarrow \alpha\text{-hydroxyethyl-TPP-E} + HCO_3^-$$

$$\alpha\text{-hydroxyethyl-TPP-E} \longrightarrow \text{Acetaldehyde} + \text{TPP-E}$$

(*b*) In the second and final step, acetaldehyde is reduced to ethanol by NADH, derived from glyceraldehyde 3-phosphate dehydrogenase reaction, *i.e.*, Step 6 of Glycolysis, through the catalytic action of *alcohol dehydrogenase* (abbreviated as ADH). This is a reversible oxidation-reduction reaction.



The yeast **alcohol dehydrogenase** (MW = 151,000) is an NAD-dependent enzyme and contains a zinc ion at its active site.

The conversion of glucose into ethanol is called alcoholic fermentation. Thus, ethanol and CO_2, instead of lactate, are the end products of this process. Alcoholic fermentation is prevented by $NaHSO_3$ which combines with acetaldehyde to give a bisulfite addition compound that is not a substrate for alcohol dehydrogenase.

The net equation for alcoholic fermentation would then be :

$$\text{Glucose} + 2\ Pi + 2\ ADP \longrightarrow 2\ \text{Ethanol} + 2\ CO_2 + 2\ ATP + 2\ H_2O$$

It may be pointed out that NAD^+ and NADH do not appear in this equation because NAD^+ generated during reduction of acetaldehyde is used in the reaction catalyzed by glyceraldehyde 3-

phosphate dehydrogenase, *i.e*., Step 6 of Glycolysis. Thus, there is no net oxidation-reduction, as in anaerobic glycolysis. Another noteworthy point of alcoholic fermentation is that there is no net change in the ratio of hydrogen to carbon atoms when D-glucose (H/C ratio = 12/6 = 2) is fermented to 2 molecule each of ethanol and CO_2 (H/C ratio = 12/6 = 2). In fact, *in all anaerobic fermentations, the H/C ratio of the reactants and products remains the same*.

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PROBLEMS

1. Xylose has the same structure as that of glucose except that it has a hydrogen atom at C-6 in place of a hydroxymethyl group. The rate of ATP hydrolysis by hexokinase is markedly enhanced by the addition of xylose. Why ?

2. The intravenous infusion of fructose into healthy volunteers leads to a two- to fivefold increase in the level of lactate in the blood, a far greater increase than that observed after the infusion of the same amount of glucose.
   (*a*) Why is glycolysis more rapid after the infusion of fructose ?
   (*b*) Fructose has been used in place of glucose for intravenous feeding. Why is this use of fructose unwise ?

3. What are the likely consequences of a genetic disorder rendering fructose 1,6-bisphosphatase in liver less sensitive to regulation by fructose 2,6-bisphosphate ?

4. If cells synthesizing glucose from lactate are exposed to CO_2 labeled with ^{14}C, what will be the distribution of label in the newly synthesized glucose ?

5. In the conversion of glucose into two molecules of lactate, the NADH generated earlier in the pathway is oxidized to NAD^+. Why is it not to the cell's advantage to simply make more NAD^+ so that the regeneration would not be necessary ? After all, the cell would save much energy because it would no longer need to synthesize lactic acid dehydrogenase.

6. People with galactosemia display central nervous system abnormalities even if galactose is eliminated from the diet. The precise reason for it is not known. Suggest a plausible explanation.

7. Write a pathway leading from glucose to lactose in mammary gland, and write a balanced equation for the overall pathway.

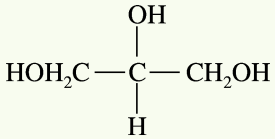
8. How many high-energy phosphates are generated in (*a*) converting 1 mole of glucose to lactate ? (*b*) converting 2 moles of lactate to glucose ?

9. Write balanced equations for all of the reactions in the catabolism of D-glucose to two molecules of D-glyceraldehyde-3-phosphate (the preparatory phase of glycolysis). For each equation, write the standard free-energy change. Then write the overall or net equation for the preparatory phase of glycolysis, including the net standard free-energy change.

10. In working skeletal muscle under anaerobic conditions, glyceraldehyde-3-phosphate is converted into pyruvate (the payoff phase of glycolysis), and the pyruvate is reduced to lactate. Write balanced equations for all of the reactions in this process, with the standard free-energy change for each. Then write the overall or net equation for the payoff phase of glycolysis (with lactate as the end product), including the net standard free-energy change.

11. The concentration of glucose in human blood plasma is maintained at about 5 mM. The concentration of free glucose inside muscle cells is much lower. Why is the concentration so low in the cell ? What happens to the glucose upon entry into the cell ?

12. Glycerol (see below) obtained from the breakdown of fat is metabolized by being converted into dihydroxyacetone phosphate, an intermediate in glycolysis, in two enzyme-catalyzed reactions. Propose a reaction sequence for the metabolism of glycerol. On which known enzyme-catalyzed reactions is your proposal based ? Write the net equation for the conversion of glycerol to pyruvate based on your scheme.



Glycerol

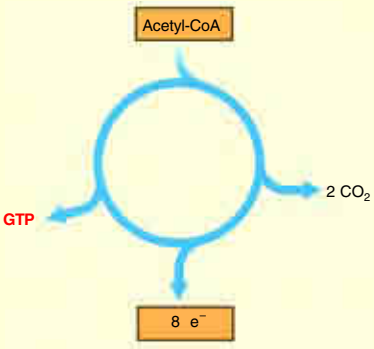
13. In muscle tissue, the rate of conversion of glycogen to glucose-6-phosphate is determined by the ratio of phosphorylase *a* (active) to phosphorylase *b* (less active). Determine what happens to the rate of glycogen breakdown if a muscle preparation containing glycogen phosphorylase is treated with (*a*) phosphorylase *b* kinase and ATP ; (*b*) phosphorylase *a* phosphatase ; (*c*) epinephrine.

14. The intracellular use of glucose and glycogen is tightly regulated at four points. In order to compare the regulation of glycolysis when oxygen is plentiful and when it is depleted, consider the utilization of glucose and glycogen by rabbit leg muscle in two physiological settings: a resting rabbit, whose leg-muscle ATP demands are low, and a rabbit who has just sighted its mortal enemy, the coyote, and dashes into its burrow at full speed. For each setting, determine the relative levels (high, intermediate, or low) of AMP, ATP, citrate, and acetyl-CoA and how these levels affect the flow of metabolites through glycolysis by regulating specific enzymes. In periods of stress, rabbit leg muscle produces much of its ATP by anaerobic glycolysis (lactate fermentation) and very little by oxidation of acetyl-CoA derived from fat breakdown.

15. Unlike the rabbit with its short dash, migratory birds require energy for extended periods of time. For example, ducks generally fly several thousand miles during their annual migration. The flight muscles of migratory birds have a high oxidative capacity and obtain the necessary ATP through the oxidation of acetyl-CoA (obtained from fats) *via* the citric acid cycle. Compare the regulation of muscle glycolysis during short-term intense activity, as in the fleeing rabbit, and during extended activity, as in the migrating duck. Why must the regulation in these two settings be different ?

16. If a cell is forced to metabolize glucose anaerobically, how much faster would glycolysis have to proceed to generate the same amount of ATP as it would get if it metabolized glucose aerobically ?

CONTENTS



Roundabouts, or traffic circles, function as hubs to facilitate traffic flow
The citric acid cycle is the biochemical hub of the cell, oxidizing carbon fuels, usually in the form of acetyl-CoA, as well as serving as a source of precursors for biosynthesis.
(Courtesy : (Upper) Chris Warren)

CHAPTER

22

Pyruvate Oxidation and Citric Acid Cycle

CELL RESPIRATION

Under aerobic conditions, the cells obtain energy from ATP, produced as a result of breakdown of glucose. However, most plant and animal cells are aerobic and hence oxidize their organic fuels (carbohydrates etc.) completely to CO_2 and H_2O. Under these conditions, the pyruvate formed during glycolysis is not reduced to lactate or ethanol and CO_2 as occurs in anaerobic conditions but instead oxidized to CO_2 and H_2O aerobically. This process is called respiration by biochemists. The biochemists use the term in a *microscopic sense* and define respiration as *a sequence of molecular processes involved on O_2 consumption and CO_2 formation by cells.*

THREE STAGES OF CELL RESPIRATION

Respiration in cells occurs in 3 stages (refer Fig. 22–1) :

1. First stage: *Oxidative decarboxylation of pyruvate to acetyl CoA and CO_2*

In this stage, the organic fuels such as carbohydrates, fatty acids and also some amino acids are oxidized to yield the 2-carbon fragments, the acetyl groups of the acetyl-coenzyme A.

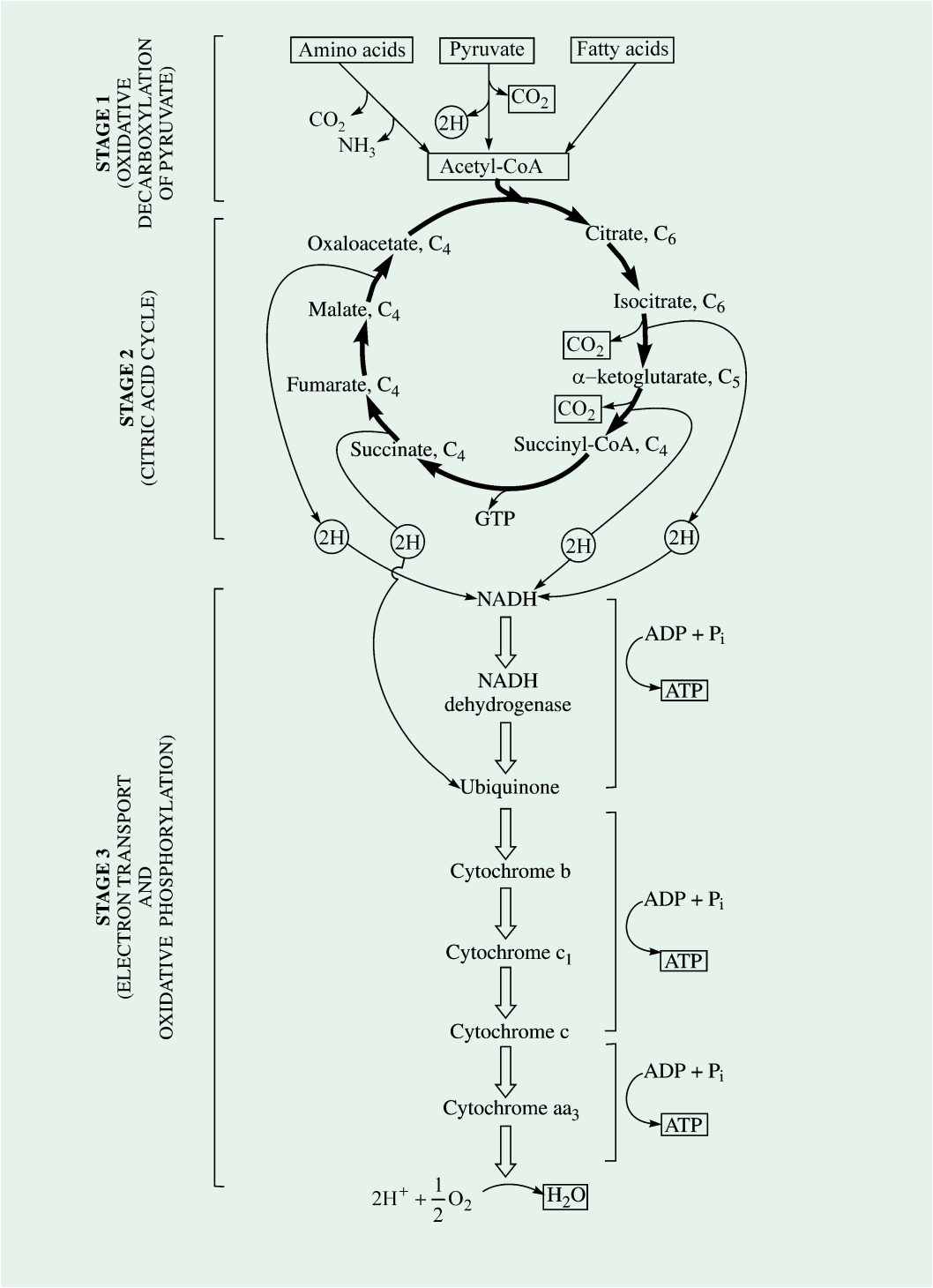


Fig. 22-1. Three stages in cell respiration

(*Adapted from Lehninger AL, 1984*)

2. Second stage: *Citric acid cycle or Acetyl CoA catabolism*

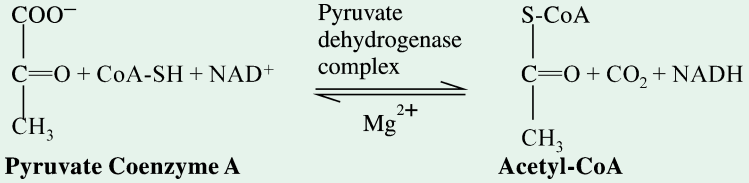
In this stage, the acetyl groups so obtained are fed into the citric acid cycle (= Krebs' cycle) which degrades them to yield energy-rich hydrogen atoms and to release CO_2, the final oxidation pruduct of organic fuels. It is, thus, the final common pathway for oxidation of fuel molecules. The cycle also provides intermediates for biosyntheses.

3. Third stage: *Electron transport and oxidative phosphorylation*

In this final stage of respiration, the hydrogen atoms are separated into protons (H^+) and energy-rich electrons. The electrons are transferred via a chain of electron-carrying molecules, the respiratory chain, to molecular oxygen, which is reduced by the electrons to form water.

Oxidative Decarboxylation of Pyruvate to Acetyl CoA

The oxidative decarboxylation of pyruvate to form acetyl-CoA is the link between glycolysis and the citric acid cycle. The reaction occurs in the mitochondrial matrix. Here, the pyruvate derived from glucose by glycolysis, is dehydrogenated to yield acetyl CoA and CO_2 by the enzyme pyruvate dehydrogenase complex (abbreviated as PDC) which is located in the matrix space of mitochondria of the eukaryotes and in the cytoplasm of the prokaryotes. The overall reaction ($\Delta G^{\circ\prime} = -8.0$ kcal/mol), catalyzed by the enzyme, is essentially irreversible and may be written as :



Pyruvate Coenzyme A **Acetyl-CoA**

Pyruvate dehydrogenase complex (Fig. 22–2) from *Escherichia coli* is a large multienzyme cluster (MW = 48,00,000 ; Pig-heart has MW = 1,00,00,000) consisting of (Table 22–1) pyruvate dehydrogenase or pyruvate decarboxylase (E_1), dihydrolipoyl transacetylase (E_2) and dihydrolipoyl dehydrogenase (E_3) and 5 coenzymes *viz.*, thiamine pyrophosphate (TPP), lipoic acid (LA), flavin adenine dinucleotide (FAD), coenzyme A (CoA) and nicotinamide adenine dinucleotide (NAD^+). Four different vitamins required in human diet are vital components of this complex enzyme. These are thiamine (in TPP), riboflavin (in FAD), pantothenic acid (in CoA) and nicotinamide (in NAD^+). Lipoic acid, however, is an essential vitamin or growth factor for many microorganisms but not so for higher animals, where it can be made from readily available precursors.

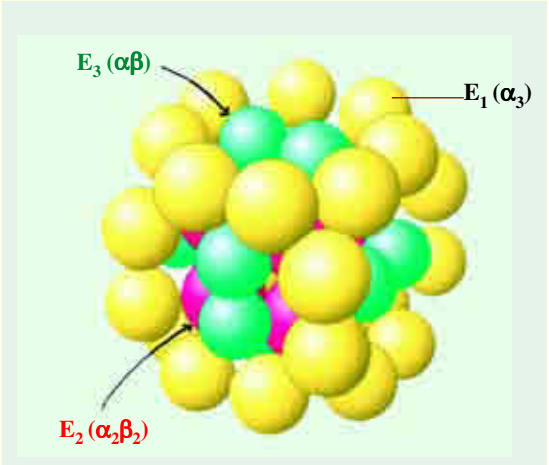


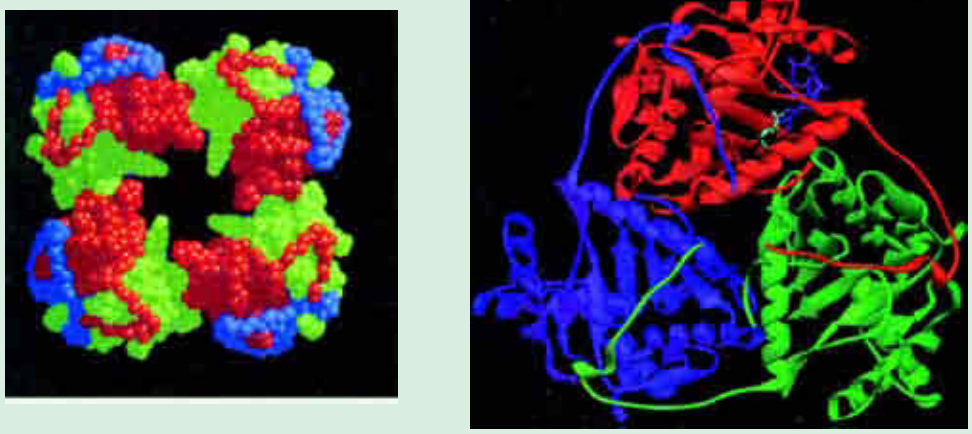
Fig. 22-2. Space-filling model of the pyruvate dehydrogenase complex

The pyruvate dehydrogenase component (E_1) is shown in (*red*), the transacetylase core (E_2) (*yellow*), and the dihydrolipoyl dehydrogenase (E_3) in (*green*).

Table 22-1. Pyruvate dehydrogenase complex of Escherichia coli

Enzyme Component	*Abbreviations*	*Prosthetic group*	*Reaction catalyzed*
Pyruvate dehydrogenase	A or E_1	TPP	Decarboxylation of pyruvate\
Dihydrolipoyl transacetylase	B or E_2	Lipoamide	Oxidation of C_2 unit and transfer to CoA
Dihydrolipoyl dehydrogenase	C or E_3	FAD	Regeneration of the oxidized form of lipoamide

This complex enzyme from *Escherichia coli* was first isloated and studied in detail by Lester J. Reed and R. M. Oliver of the University of Texas in 1974. Its molecule exhibits a distinct polyhedral appearance with a diameter of 350 ± 50 Å and a height of 225 ± 25 Å. The 'core' of the multienzyme cluster is occupied by dihydrolipoyl transacetylase (E_2) which consists of 24 polypeptide chain subunits, each containing two lipoic acid groups in amide linkage with the ε-amino groups of specific lysine residues in the active sites of the subunits. The 3-'D' structure of dihydrolipoyl transacetylase (E_2) catalytic domain is presented in Fig. 22–3. The other two enzyme components (pyruvate dehydrogenase and dihydrolipoyl dehydrogenase) are attachced to the outside of dihydrolipoyl transacetylase core. Pyurvate dehydrogenase contains bound TPP



(*a*) (*b*)

Fig. 22–3. The x-ray structure of the *A. vinelandii* dihydrolipoyl transacetylase (E_2) catalytic domain

(*a*) *A space-filling drawing*. Each residue is represented by a sphere centred on its Cα atom. The 8 trimers (24 identical subunits) are arranged at the corners of a cube as viewed along one of the cube's 4-fold axes (only the forward half of the complex is visible). The edge length of the cube is ~ 125 Å. Note that the subunits in a trimer are extensively associated, but that the interactions between contacting trimers are relatively tenuous.

(*b*) Ribbon diagram of a trimer as viewed along its 3-fold axis (along the cube's body diagonal) from outside the complex. Coenzyme A (*purple*) and lipoamide (*light blue*), in skeletal form, are shown bound in the active site of the red subunit. Note how the N-terminal "elbow" of each subunit extends over a neighbouring subunit; its deletion greatly destabilizes the complex.

(From Wim Hol, University of Washington)

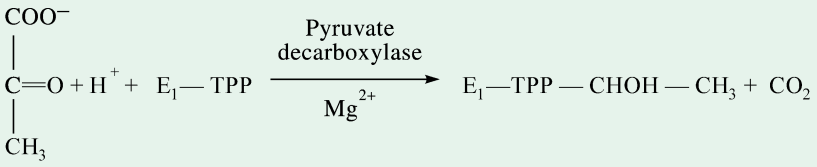
and dihydrolipoyl dehydrogenase contains bound FAD. The lipoyllysyl groups of the 'core' enzyme serve as `swinging or flexible arms' that can transfer hydrogen atoms and acetyl groups from one enzyme molecule to another in the multienzyme complex. All these enzymes and coenzymes are organized into a cluster to keep the prosthetic groups close together, thus allowing the reaction intermediates to react quickly with each other and also minimizing the side reactions.

The constituent polypeptide chains of the complex are held together by noncovalent forces. At alkaline pH, the complex dissociates into the pyurvate dehydrogenase component and a subcomplex of the other two enzymes. The transacetylase can then be separated from the dehydrogenase at neutral pH in the presence of urea. These 3 enzyme components associate to form the pyruvate dehydrogenase complex when they are mixed at neutral pH in the absence of urea.

There are 5 successive stages in the conversion of pyruvate into acetyl CoA. These are as follows :

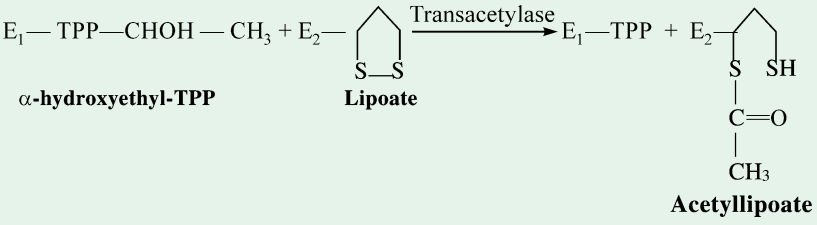
Stage 1 :

Pyruvate loses its carboxyl group as it reacts with the bound TPP of pyruvate dehydrogenase (E_1) to form the hydroxyethyl derivative of thiazole ring of TPP.



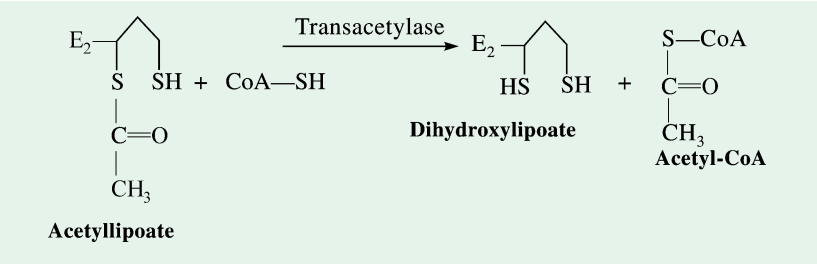
Stage 2 :

The H atoms and acetyl group from TPP is transferred to the oxidized form of lipoyllysyl groups of the 'core' enzyme E_2 to form the 6-acetyl thioester of the reduced lipoyl groups.



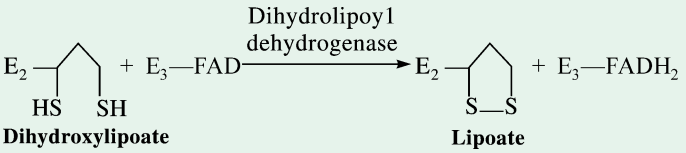
Stage 3 :

A molecule of CoA-SH reacts with the acetyl derivative of E_2 to produce acetyl-S-CoA and the fully reduced (or dithiol) form of lipoyl groups.



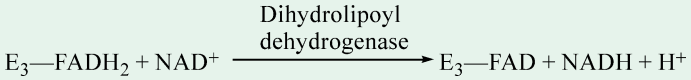
Stage 4 :

The fully reduced form of E_2 is acted upon by E_3 which promotes transfer of H atoms from the reduced lipoyl groups to the FAD prosthetic group of E_3.



Stage 5 :

In this last stage, the reduced FAD group of E_3 transfers hydrogen to NAD^+, forming NADH



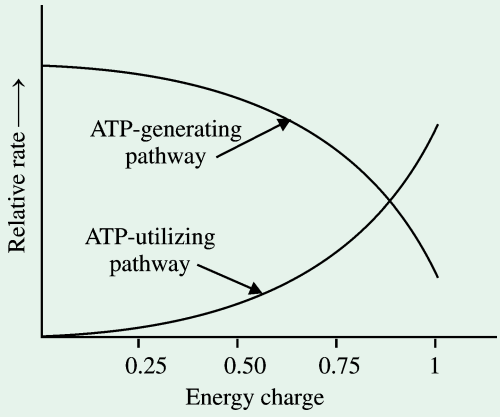
Regulation of Oxidative Decarboxylation of Pyruvate

The conversion of pyruvate into acetyl-CoA is a key irreversible step in the metabolism of animals becaause the animals cannot convert acetyl-CoA into glucose. The carbon atoms of glucose has two fates : (*a*) oxidation of CO_2 *via* the citric acid cycle and (*b*) incorporation into lipid. Therefore, it seems that pyruvate dehydrogenase complex (PDC) which catalyzes oxidative

Energy charge (reference on the next page), a term coined by Daniel Atkinson in 1970, is the energy status of the cell. In mathematical terms, Atkinson defined energy charge as that fraction of the adenylic system (ATP + ADP + AMP) which is composed of ATP. In other words, it is proportional to the mole fraction of ATP plus half the mole fraction of ADP, given that ATP contains two and ADP contains one anhydride bond. Hence, the energy charge is given by the equation :

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The energy charge is one if the total adenine nucleotide pool is fully phosphorylated to ATP (*i.e.*, it is all ATP) and zero if the adenine nucleotides are fully `empty' and present only as AMP (*i.e.*, it is all AMP). The energy charge may have values ranging between these two extremes. Normally, the energy charge of cells ranges between 0.80 and 0.95 which means that adenylate system is almost completely charged. A high energy charge inhibits all ATP-generating (*i.e.*, catabolic) pathways but stimulates the ATP-utilizing (*i.e.*, anabolic) pathways.



Energy charge and the metabolic rate

High concentrations of ATP inhibit the relative rates of a typical ATP-generating (= catabolic) pathway and stimulate that of the typical ATP-utilizing (= anabolic) pathway.

In plots of the reaction rates of such pathways versus the energy charge, the curves are steep near an energy charge of 0.9, where they usually intersect (adjoining figure). It is evident that the control of these pathways has evolved to maintain the energy charge within rather narrow limits. In other words, *the energy charge, like the pH of a cell, is buffered.*

An alternative index of the energy status is the ***phosphorylation potential,*** which is given by the equation:

$$\text{Phosphorylation potential} = \frac{[\text{ATP}]}{[\text{ADP}][\text{Pi}]}$$

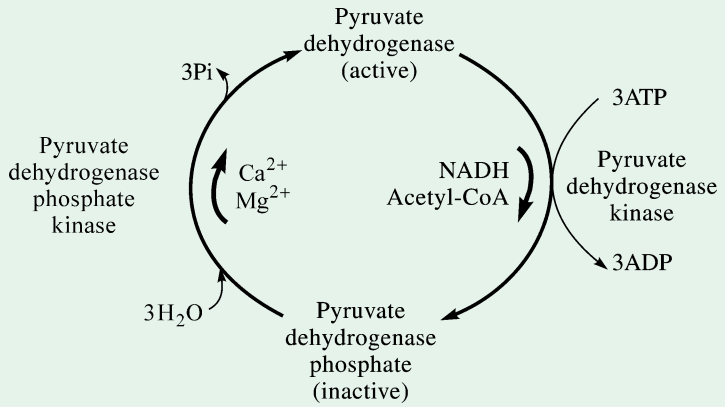
The phosphorylation potential, contrary to the energy charge, depends on the concentration of Pi and is directly related to the free energy-storage available from ATP.

decarboxylation of pyruvate, has a very stringent regulatory mechanism. This complex enzyme system is regulated in 3 ways :

A. End-product inhibition. Acetyl-CoA and NADH, both end products of the pyruvate dehydrogenase reaction, are potent allosteric inhibitors of the enzyme. Acetyl-CoA inhibits the transacetylase component whereas NADH inhibits the dihydrolipoyl dehydrogenase component. The inhibitory effects are reversed on addition of coenzyme A and NAD^+ respectively.

B. Feedback regulation. The activity of PDC is controlled by the *energy charge*. The pyruvate dehydrogenase component is specifically inhibited by GTP and activated by AMP.

C. Covalent modification. Under conditions of high concentrations of ATP, acetyl-CoA and those of the cycle intermediates, further formation of acetyl-CoA is slowed down. This is accomplished by what is called *covalent modification*. ATP inactivates pyruvate dehydrogenase complex (PDC) by phosphorylating 3 different serine residues of its pyruvate dehydrogenase component in the presence of an auxiliary enzyme, *pyruvate dehydrogenase kinase*. NADH, ATP and acetyl-CoA stimulate the rate of phosphorylation whereas pyruvate, Ca^{2+} and K^+ inhibit phosphorylation. However, if the demand for ATP increases causing ATP level to decline, the inactive enzyme complex becomes active again by the hydrolytic removal of phosphate group from its pyruvate dehydrogenase component in the presence of another enzyme, *pyruvate dehydrogenase phosphate kinase*. Dephosphorylation is enhanced by high levels of pyruvate, Ca^{2+} and Mg^{2+}. Both pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate kinase are also present in PDC.



Sir HANS ADOLF KREBS (LT, 1900–1981)

A German-born and trained British biochemist, was one of the outstanding scientists of the century. From 1926 to 1930, he worked in Berlin with Otto Warburg, himself one of the great pioneers of modern biochemistry. In 1932, Krebs worked out the outlines of the urea cycle with a medical student Kurt Henseleit at the University of Freiburg. In 1933, he emigrated to England to the University of Cambridge. Later, he moved to the University of Shefield, where he worked out a major part of the citric acid cycle using mainly the pigeon breast muscles, a very actively respiring tissue. In 1954, he became head of the Biochemistry Department at Oxford. On his retirement from that position in 1967, he engaged himself in examining the regulation of metabolism in the Department of Medicine, at Oxford until his death. Krebs shared the coveted **Nobel Prize for Physiology and Medicine** in 1953 with **Fritz Albert Lipmann**, the 'father' of ATP cycle, for their work in intermediary metabolism. It is of interest to note that when Krebs' original manuscript on TCA cycle was submitted for publication, it was rejected because of a lack of publishing space.

CITRIC ACID CYCLE OR KREBS CYCLE OR ACETYL-COA CATABOLISM

The most nearly universal pathway for aerobic metabolism is the cyclic series of reactions, termed *citric acid cycle* (CAC) or *Krebs cycle*. The first name has been applied because citric acid (Fig. 22–4) is the first intermediate formed in this cycle. The second name has been given in honour of its most illustrious proponent, Sir Hans A. Krebs, who first postulated it in 1937 and the cycle has since then been slightly modified in a form we know today. However, a third name *tricarboxylic acid cycle* (TCA) was given to it some years after Krebs postulated the cycle because it was then not certain whether citric acid or some other tricarboxylic acid (*e.g.*, isocitric acid) was first product of the cycle. Since we now know with certainty that citric acid is indeed the first tricarboxylic acid formed, the use of the term `tricarboxylic acid cycle' is not appropriate and hence be discouraged. *This cycle forms the hub of metabolism of almost all cells* and has truly been regarded as the most important single discovery in the history of metabolic biochemistry. In fact, more of the details have been worked out and more of the ramifications explored for the citric acid cycle than for any other pathway, except perhaps glycolysis.

Fig. 22–4. Crystals of citric acid, as viewed under polarized

The citric acid cycle is a series of reactions in mitochondria that bring about the complete oxidation of acetyl-CoA to CO_2 and liberate hydrogen equivalents which ultimately form water. This cyclic sequence of reactions provides electrons to the transport system, which reduces oxygen while generating ATP. *The citric acid cycle is the final common pathway for the oxidation of fuel molecules– amino acids, fatty acids and carbohydrates*. Most fuel molecules enter the cycle as acetyl-CoA. Two fundamental *differences* exist between glycolysis and citric acid cycle:

1. Glycolysis takes place by a *linear* sequence of 10 enzyme—catalyzed reactions. In contrast, citric acid cycle proceeds in a *cyclic* way by 8 enzyme-catalyzed reactions.
2. The reactions of glycolysis occur in the *cytosol* in contrast with those of citric acid cycle which occur inside *mitochondria*.

ENZYMES INVOLVED IN THE CITRIC ACID CYCLE

A landmark discovery was made in 1948 by Eugene P. Kennedy and Albert L. Lehninger when they found that rat liver mitochondria could catalyze the oxidation of pyruvate and all the intermediates of the citric acid cycle by

ALBERT L. LEHNINGER (LT,1917–1986)

Lehninger was an, American biochemist. He, along with Kennedy, discovered, in 1948, that the enzymes involved in the citric acid and respiratory cycles of energy transformation in the cell are located in mitochondria. With regard to the regulation of enzyme activity, he held the view :

"Living cell are self-regulating chemical engines, timed to operate on the principle of maximum economy."

He also wrote a very famous, self-explanatory and informative text '*Principles of Biochemistry*', first published in 1982 which transformed the teaching of biochemistry.

Table 22–2. Enzymes of the citric acid cycle

*Step No.**	*Enzyme*	*Location (in mitochondria)*	*Coenzymes(s) and Cofactor(s)*	*Inhibitor(s) (kcal/mol)*	*Type of Reaction Catalysed*	*$\Delta G^{\circ\prime}$ (kcal/mol)*
1.	Citrate synthase	Matrix space	CoA	Monofluoroacetyl-CoA	Condensation	–7.7
2.	Aconitase	Inner membrane	Fe^{2+}	Fluoroacetate	Isomerization	+1.59
3.	Isocitrate dehydrogenase	Matrix space	NAD^+, $NADP^+$ Mg^{2+}. Mn^{2+}	ATP	Oxidative decarboxylation	–2.0
4.	α-ketoglutarate dehydrogenase complex	Matrix space	TPP, LA, FAD, CoA, NAD^+	Aresenite, Succinyl-CoA NADH	Oxidative decarboxylation	–8.0
5.	Succinyl-CoA synthetase	Matrix space	CoA	—	Substrate-level phosphorylation	–7.0
6.	Succinate dehydrogenase	Inner membrane	FAD	Malonate, Oxaloacetate	Oxidation	~0
7.	Fumarase	Matrix space	None	—	Hydration	≈0
8.	Malate dehydrogenase	Matrix space	NAD^+	NADH	Oxidation	+7.1

*The numbers in this column correspond to the reactions as described in the text and catalyzed by the enzyme specified.

Contents

molecular oxygen. Since only Mg^{2+} and an adenylic acid (ATP, ADP or AMP) had to be added, this finding meant that mitochondria contain not only all the enzymes and coenzymes required for the citric acid cycle but also those needed to transport the electrons from the substrate to molecular oxygen. Later work has shown that some enzymes (such as aconitase, fumarase and malate dehydrogenase), required in the cycle, are also found in the cytoplasm, but the reactions they catalyze are independent of the mitochondrial oxidation process. The different enzymes involved in the citric acid cycle are located either in the inner membrane or in the matrix space of the mitochondria (Fig. 22–5).

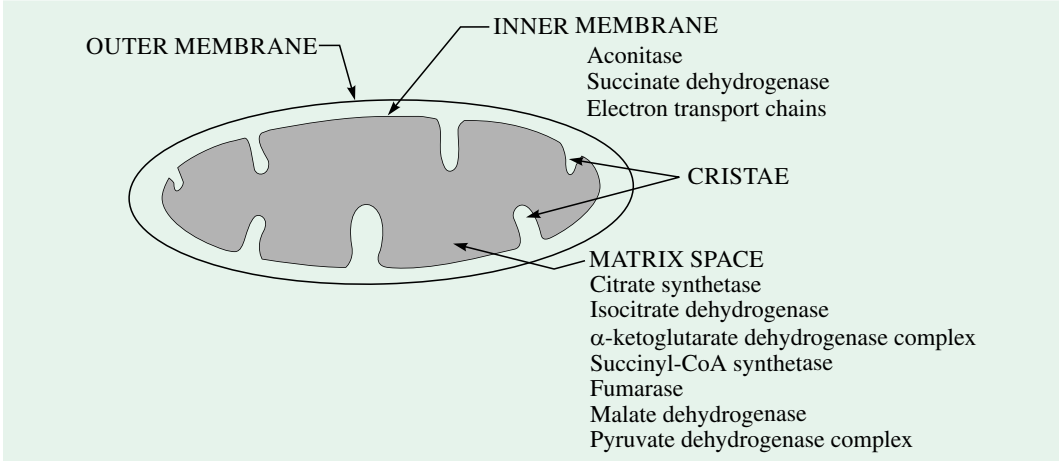


Fig. 22–5. Location of citric acid cycle enzymes in mitochondria

The various enzymes involved in different reactions of the cycle are presented in Table 22–2 along with their characteristics.

OVERVIEW OF THE CITRIC ACID CYCLE

An overall pattern of the citric acid cycle is represented in Fig. 22–4. To begin with, acetyl-CoA donates its 2-carbon acetyl group to the 4-carbon tricarboxylic acid, **oxaloacetate** to form a 6-carbon tricarboxylic acid, **citrate.** Citrate is then transformed into another 6-carbon tricarboxylic acid, **isocitrate.** Isocitrate is then dehydrogenated with the loss of CO_2 (*i.e.*, oxidatively decarboxylated) to yield the 5-carbon dicarboxylic acid, **α-ketoglutarate.** The latter compound undergoes further oxidative decarboxylation to yield the 4-carbon dicarboxylic acid, succinate and to release a second molecule of CO_2. Succinate then undergoes three successive enzyme-catalyzed reactions to regenerate oxaloacetate, with which the cycle began. Thus, oxalocetate is regenerated after one turn of the cycle and is now ready to react with another molecule of acetyl-CoA to start a second turn.

> As per Circular No 200 of the Committee of Editors of Biochemical Journals Recommendations (1975), there is a standard biochemical convention, according to which the names of the various carboxylic acids are written by adding the suffix **—ate** in the name of the acid mentioned, as it is taken to mean any mixture of free acid and the ionized form(s) (according to pH) in which the cations are not specified.

In each turn of the cycle, one acetyl group (*i.e.*, 2 carbons) enters as acetyl-CoA and 2 moles of CO_2 are released. In each turn, one mole of oxaloacetate is used to form citrate but after a series of reactions, the oxaloacetate is regenerated again. Therefore, no net removal of oxaloacetate occurs when the citric acid cycle operates and one mole of oxaloacetate can, theoretically, be sufficient to bring about oxidation of an indefinite number of acetyl groups.

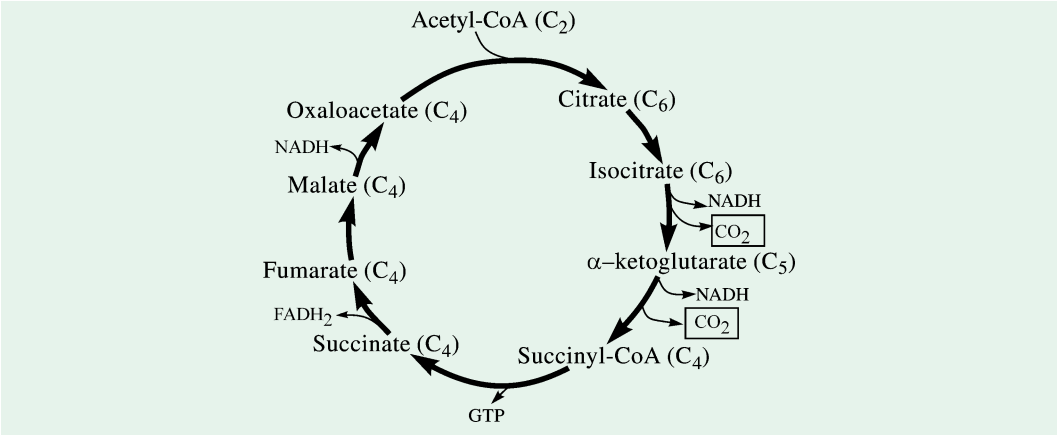


Fig. 22-6. An outline of the citric acid cycle

In bracket, the number of carbon atoms in each intermediate is shown. However, succinyl-CoA has 4 carbon atoms in its succinyl groups, the portion of the molecule which is converted into free succinate.

Another diagram (Fig 22–7 akin to Fig. 22-6, shows the positions of various intermediate products, whether drawn off or replenished in the citric acid cycle.

REACTION STEPS OF THE CITRIC ACID CYCLE

The citric acid cycle proper consists of a total of 8 successive reaction steps,. each of which is catalyzed by an enzyme. The details of these reactions and those of the enzymes which catalyze them are given below :

Step 1 : *Condensation of acetyl-CoA with oxaloacetate*

For oxaloacetate, some biochemical journals use the spelling, **'oxalacetate'.**

The cycle starts with the joining of a 4-carbon unit, oxaloacetate (OAA) and a 2-carbon unit,the acetyl group of acetyl-CoA. Oxaloacetate reacts with acetyl-CoA plus water to yield a C_6 compound, citrate plus coenzyme A in the presence of a regulatory enzyme, **citrate**

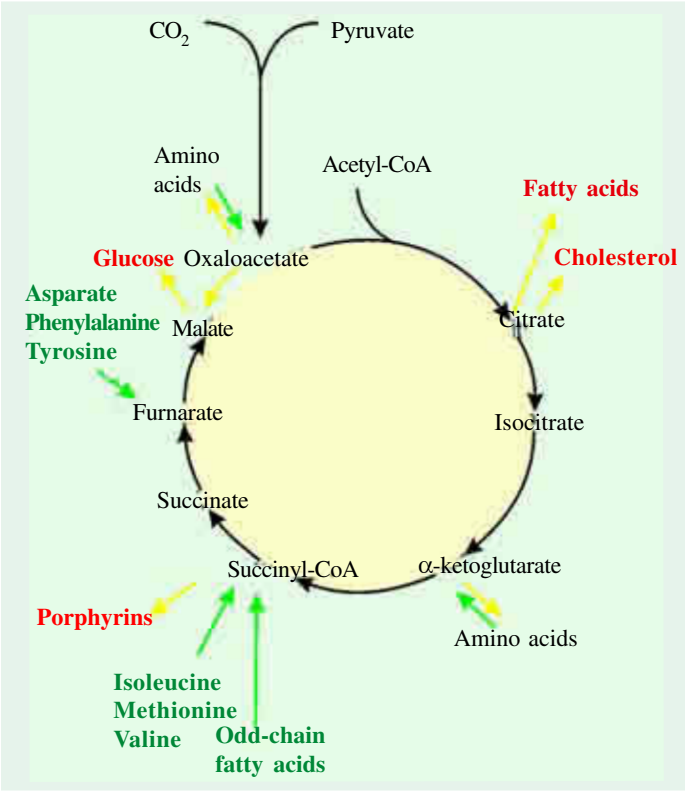
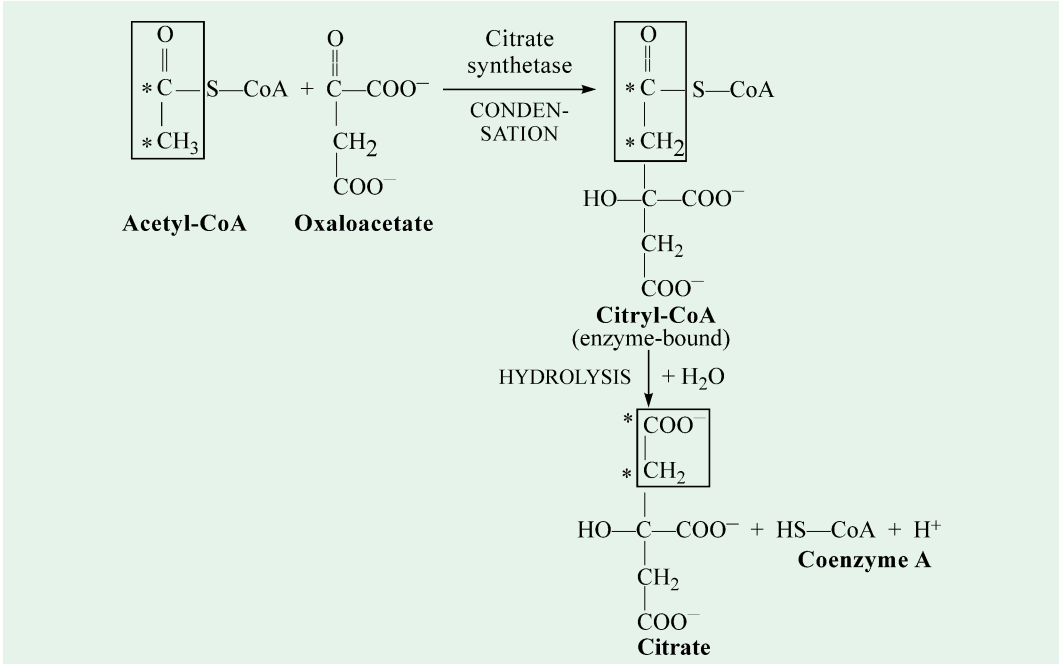


Fig. 22–7. A diagram of the citric acid cycle, indicating the positions at which intermediates are drawn off for use in anabolic pathways *(red arrows)* **and the points where anaplerotic reactions replenish depleted cycle intermediates** *(green arrows)***.**

Reactions involving amino acid transamination and deamination are reversible, so their direction varies with metabolic demand.

synthase (variously called as *condensing enzyme* or *citrate oxaloacetate lyase* (*CoA acetylating or citrogenase*). The enzyme condenses the methyl carbon of acetyl group of acetyl-CoA and the carbonyl carbon of oxaloacetate forming a transient intermediate compound, citryl-CoA (which is the coenzyme A thiol-ester of citric acid) on the active site of the enzyme. Citryl-CoA then



undergoes hydrolysis so that its thiol ester bond is cleaved to set free coenzyme A and to form citrate. Both coenzyme A and citrate are then released from the active site of the enzyme. Thus, this reaction is an aldol condensation followed by hydrolysis. The overall reaction has a large negative standard free energy of hydrolysis (−7.7 kcal/mol). Therefore, the reaction proceeds far to the right, *i.e.*, in the direction of citrate synthesis. The coenzyme A formed in this reaction is now free to participate in the oxidative decarboxylation of another molecule of acetyl-CoA for entry into the citric acid cycle.

The mammalian **citrate synthase** (MW = 89,000) consists of two identical subunits

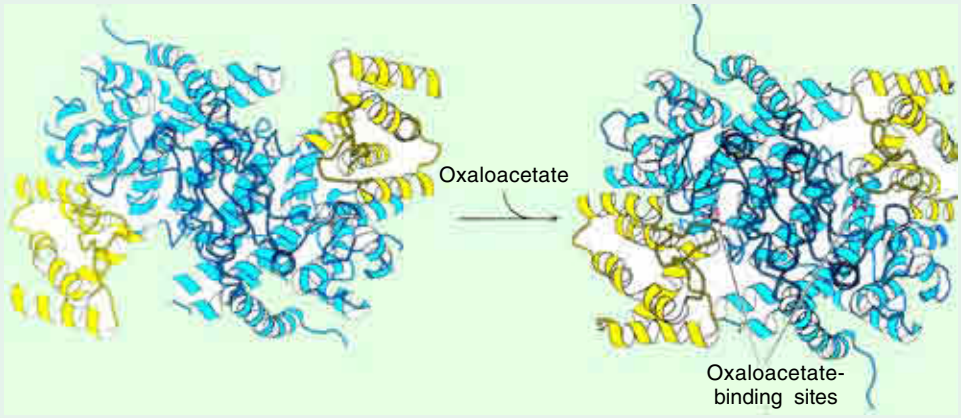


Fig. 22–8. Ribbon model of the conformational changes in citrate synthase on binding oxaloacetate The small domain of each subunit of the homodimer is shown in *yellow*, the large domain is shown in *blue* (*left*) Open form of enzyme alone. (*Right*) Closed form of the liganded enzyme.

(Figs. 22-9) and is exceptionally stable. Its specificity for the two substrates (acetyl-CoA and oxaloacetate) is quite stringent, with monofluoroacetyl-CoA as the only alternate reactant. This compound in the presence of oxaloacetate is converted to fluorocitrate at about 1/10th the rate of the natural substrate.

Step 2 : *Isomerization of citrate into isocitrate*

Citrate has a tertiary alcohol group which could not be attacked without breaking a carbon bond, but rearrangement into the isomer, isocitrate creates a secondary alcohol group that can be oxidized. Therefore, for further metabolism, citrate must be converted into isocitrate. This conversion is accomplished by this step of the cycle. Here the citrate is isomerized into isocitrate through the intermediary formation of the tricarboxylic acid, cis-aconitate. The isomerization takes place in 2 stages:

(*a*) *dehydration* of citrate to cis-aconitate, which remains bound to the enzyme, and

(*b*) *rehydration* of cis-aconitate to isocitrate.

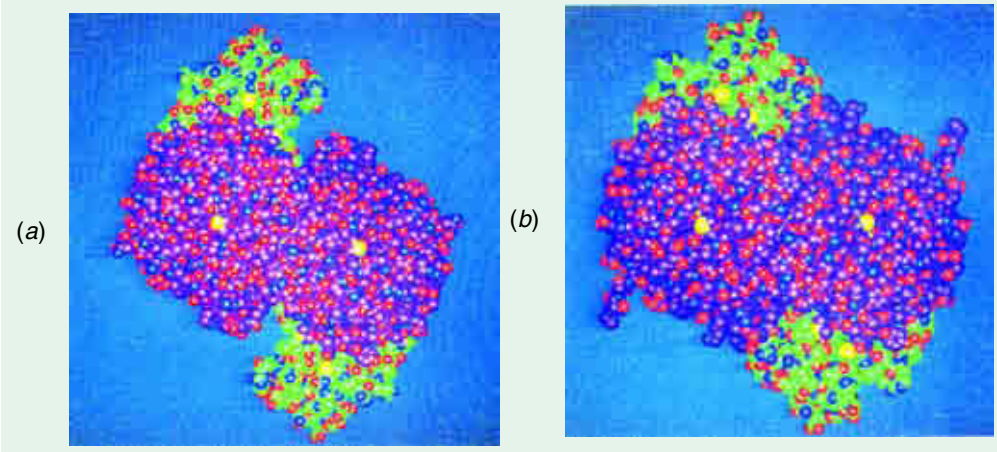
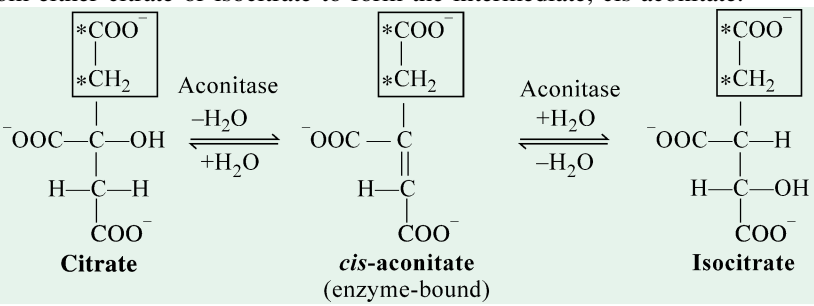


Fig. 22–9. A space-filling drawing of citrate synthase in (*a*) the open conformation and (*b*) the closed, substrate-binding conformation

The C atoms of the small domain in each subunit of the enzyme are *green* and those of the large domain are *purple*, N, O, and S atoms in both domains are *blue, red,* and *yellow*. The view is along the homodimeric protein's twofold rotation axis. The large conformational shift between the open and closed forms entails relative interatomic movements of up to 15 Å.

(Based on X-Ray structures determined by James Remington and Robert Huber)

The result is an interchange of an H and OH. Both the reactions are reversible and are catalyzed by the same enzyme, *aconitase* (= *aconitate hydratase*). When aconitase catalyzes the addition of water to the double bond of cis-aconitate, the OH group may be added to either carbon; in one case citrate is formed and in the other, isocitrate. The enzyme also catalyzes the removal of water from either citrate or isocitrate to form the intermediate, cis-aconitate.



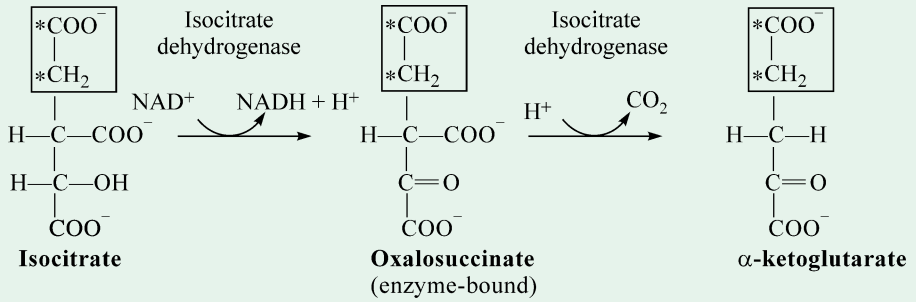
It is possible that cis-aconitate may not be an obligatory intermediate between citrate and isocitrate but may, in fact, be a side branch from the main pathway.

Experiments using ^{14}C-labelled intermediates indicate that citrate, although being a symmetrical molecule, reacts in an asymmetrical manner and that the *aconitase always acts on that part of the citrate molecule which is derived from oxaloacetate*. The standard free energy change of the overall reaction (+ 1.59 kcal/mol) is small enough so that the reaction can go in either direction. Although the equilibrium mixture (at pH 7.4 and 25°C) contains about 90% citrate, 4% *cis*-aconitate and 6% isocitrate, the reaction in the cell is driven to the right (*i.e.*, towards isocitrate formation) because the product isocitrate is rapidly transformed in the subsequent step of the cycle.

Aconitase is a rather complex enzyme. Pig heart aconitase (MW = 89,000) is a dimer of identical units, each containing iron and sulfur atoms arranged in a cluster called iron-sulfur centre. However, aconitase differs from other enzymes of its own category, *i.e.*, hydratases (= enzymes catalyzing the reversible hydration of double bonds) in that they lack such an iron-sulfur centre. The iron-sulfur centre presumably acts as a prosthetic group, although its precise function is still not known. The presence of such a centre, however, is baffling because there occurs no transfer of electrons during the reaction. The reaction is inhibited by fluoroacetate which, in the form of fluoroacetyl-CoA, condenses with oxaloacetate to form fluorocitrate. The latter inhibits aconitase and thus prevents utilization of citrate. Fluoroacetate occurs naturally in the leaves of a South African plant, *Dichopetalum cymosum* which is toxic to animals that feed on it.

Step 3 : *Oxidative decarboxylation of isocitrate*

This is the first of the 4 oxidation-reduction reactions in the citric acid cycle. Isocitrate is oxidatively decarboxylated to a C_5 compound, α-ketoglutarate (= 2-oxoglutarate) through the intermediary formation of a tricarboxylic keto acid, oxalosuccinate. The reaction takes place in 2 stages : (*a*) *dehydrogenation* of isocitrate to oxalosuccinate which remains bound to the enzyme, and (*b*) *decarboxylation* of oxalosuccinate to a-ketoglutarate. NAD^+ or $NADP^+$ is required as



electron acceptor in the first stage. Both the reactions are irreversible and are catalyzed by the same enzyme, *isocitrate dehydrogenase*. Equilibrium favours α-ketoglutarate formation as, under physiologic conditions, the $\Delta G^{\circ\prime}$ is equal to –5 kcal/mol (*i.e.*, a large negative value). This is the first '*committed step*' in the Krebs cycle as it has no other function than to participate in the cycle.

It may be noticed that the carbonyl group of the intermediate oxalosuccinate is in the β position relative to the middle carboxylate group (The carbonyl group is also in the α-position with respect to the top carboxylate group, but that is not relevant to our point). And we know from the knowledge of organic chemistry that β-keto acids are easily decarboxylated and oxalosuccinate does it.

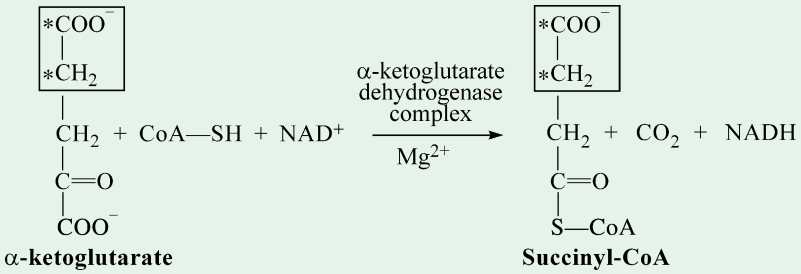
There are 2 types of **isocitrate dehydrogenase**, one requiring NAD^+ as electron acceptor (*NAD^+-specific*) and the other requiring *$NADP^+$* ($NADP^+$-*specific*). Both the types appear to participate in the citric acid cycle, but the NAD^+-specific isocitrate dehydrogenase is predominant. The NAD^+-specific enzyme is found only in mitochondria, whereas the $NADP^+$-specific enzyme is located in both mitochondria and the cytosol. Both the enzymes require the divalent metal ions (Mg^{2+} or Mn^{2+}) for activity, like many enzymes that catalyze β-decarboxylations. Enzymes catalyzing

dehydrogenations without decarboxylation do not require metallic ions for activity, however. The mitochondrial NAD^+-specific enzyme (MW = 16,000) has 3 different subunits present in a ratio of 2 : 1 : 1. Each molecule of enzyme has two binding sites, each for metal ion, isocitrate and NAD+. This enzyme is markedly activated by ADP which lowers Km for isocitrate and is inhibited by NADH and NADPH.

The $NADP^+$-specific (or $NADP^+$-dependent) enzymes show none of these peculiar properties. They also differ from the NAD^+-dependent enzymes in their size and in the nature of the reaction catalyzed. Furthermore, the $NADP^+$-specific enzyme also oxidizes oxalosuccinate when added to the system. On the contrary, the $NADP^+$-specific enzyme, which functions as an integral component of the citric acid cycle, does not decarboxylate added oxalosuccinate. The metabolic significance of $NADP^+$-specific enzyme lies not in the operation of the citric acid cycle but as a source of reducing equivalents.

Step 4 : *Oxidative decarboxylation of a-ketoglutarate*

One of the peculiarities of the citric acid cycle is that *it contains two successive oxidative decarboxylation steps* (*Steps 3 and 4*) *of quite different reaction types*. In this step, α-ketoglutarate is oxidatively decarboxylated, in a manner analogous to the oxidative decarboxylation of pyruvate, to form a C_4 thiol ester, succinyl-CoA and CO_2 by the enzyme *α-ketoglutarate dehydrogenase complex* (α-KDC) which is located in the mitochondrial space. The reaction has a high negative value of $\Delta G^{\circ\prime}$ (– 8.0 kcal/mol), and is therefore physiologically irreversible and proceeds far to the right. The high negative $\Delta G^{\circ\prime}$ value is sufficient for the creation of a high energy bond in succinyl-CoA.



The reaction is virtually identical to the pyruvate dehydrogenase complex (PDC) reaction in that both promote the oxidation of an a-keto acid with loss of the carboxyl group as CO_2. However, there exists an important difference between the two : *the α-KDC system does not have so elaborate a regulatory mechanism as PDC system.*

The immediately preceding isocitrate dehydrogenase reaction (*i.e.*, Step 3) is also an oxidative decarboxylation, but of a 3-hydroxycarboxylate. Moreover, the standard free energy change value of this reaction (–5 kcal/mol) is too less to support the formation of an extra high-energy bond (which is formed in Step 4) even at low CO_2 concentration in the tissues.

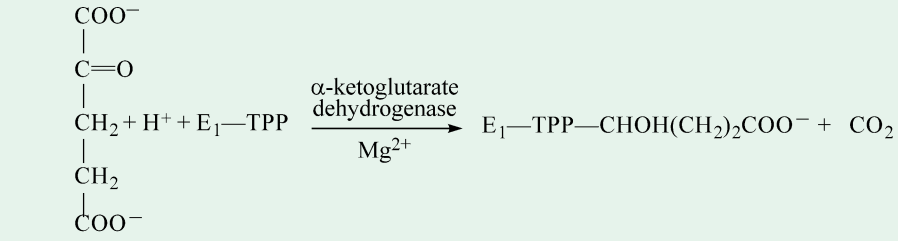
The **α-ketoglutarate dehydrogenase complex** from pig heart (MW = 33,00,000) is much smaller than the PDC from the same source (MW = 1,00,00,000). The pig heart α-KDC is a large multienzyme cluster consisting of 3 enzyme components, *viz.*, α-ketoglutarate dehydrogenase or α-ketoglutarate decarboxylase (12 moles per mole of the complex), transsuccinylase (24 moles) and dihydrolipoyl dehydrogenase (12 moles). The complex also requires the same 5 coenzymes, as required by pyruvate dehydrogenase complex, for activity, *viz.*, thiamine pyrophosphate (6 moles), lipoic and (6 moles), flavine adenine dinucleotide (8 moles), coenzyme A and nicotinamide adenine dinucleotide. The compelx enzyme molecule is comparable in size to ribosomes.

The transsuccinylase (B′) component, like the transacetylase of PDC, forms the 'core' of the multienzyme complex while the α-ketoglutarate dehydrogenase (A′) and dihydrolipoyl dehydrogenase (C′) components are arranged on the periphery,. Again, A′ binds to B′ and B′ binds to C′ but A′ does not bind directly to C′ . The α-ketoglutarate dehydrogenase (A′) and transsuccinylase (B′)

components are different from the corresponding components (A and B) in the pyruvate dehydrogenase complex. However, the dihydrolipoyl dehydrogenase components (C and C′) of the two enzyme complexes are similar. Lipoic acid is attached to the core transsuccinylase by forming an amide bond with lysine side chains. This places the reactive disulfide groups (—S—S—) at the end of a long flexible chain. The ability of the chain to swing the disulfide group in contact with the different proteins is an important feature of the enzyme complex. As in the case of pyruvate oxidation, *arsenite* inhibits the reaction, causing the substrate α-ketoglutarate to accumulate. The α-KDC is inhibited by both succinyl-CoA and NADH, the former being more effective.

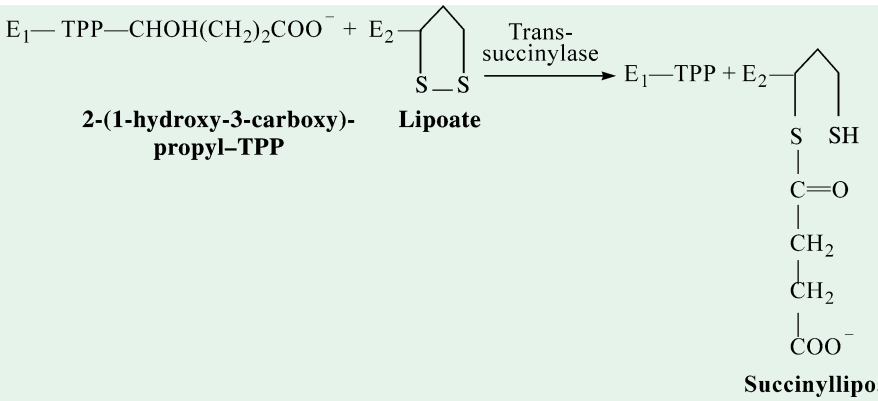
There are 5 successive stages in the conversion of a-ketoglutarate to succinyl-CoA. These are as follows :

Stage 1. α-ketoglutarate loses its carboxyl group as it reacts with the bound TPP of a-ketoglutarate dehydrogenase (E_1) to form the hydroxy-carboxypropyl derivative of thiazole ring of TPP.



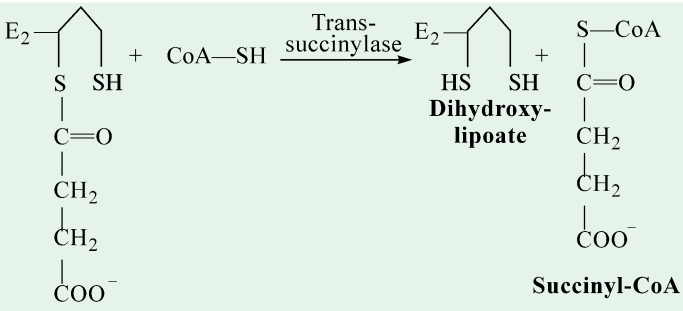
α-ketoglutarate **2-(1-hydroxy-3-carboxy)-propy1–TPP**

Stage 2. The H atoms and succinyl group from TPP is transferred to the oxidized form of lipoyllysyl groups of the 'core' enzyme E_2 to form the succinyl thioester of the reduced lipoyl groups.



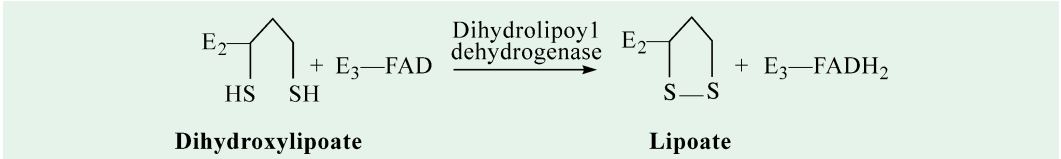
2-(1-hydroxy-3-carboxy)-propyl–TPP **Lipoate** **Succinyllipoate**

Stage 3. A molecule of CoA—SH reacts with the succinyl derivative of E_2 to produce succinyl-S-CoA and the fully reduced (or dithiol) form of lipoyl groups.

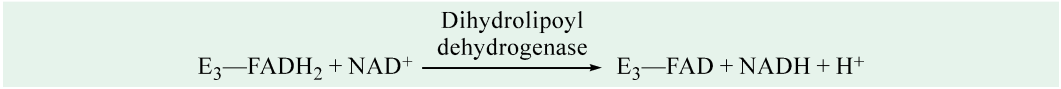


Succinyllipoate **Dihydroxy-lipoate** **Succinyl-CoA**

Stage 4. The fully reduced form of E_2 is acted upon by E_3 which promotes transfer to H atoms from the reduced lipoyl groups to the FAD prosthetic group of E_3.



Stage 5. In this last stage, the reduced FAD group of E_3 transfers hydrogen to NAD^+, forming NADH.



Step 5 : *Conversion of succinyl-CoA into succinate*

While acetyl-CoA can undergo a wide variety of metabolic reactions, the fate of succinyl- CoA is much more limited. Its main route is to continue in the cycle. Succinyl-CoA is a high- energy compound. Like acetyl-CoA, it has a strong negative $\Delta G^{\circ\prime}$ value (–8.0 kcal/mol) for hydrolysis of the thioester bond:

$$\text{Succinyl-S-CoA} + H_2O \longrightarrow \text{Succinate} + \text{CoA—SH} + H^+$$

But such a simple hydrolysis of succinyl-CoA in cells does not occur, as it would mean wastage of free energy. Instead, succinyl-CoA undergoes an energy-conserving reaction in which the cleavage of its thioester bond is accompanied by the phosphorylation of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). The reaction is catalyzed by *succinyl-CoA synthase* (= *succinic thiokinase*). Its structural details are presented in Fig. 22–10. The enzyme involves the formation of an intermediate, succinyl phosphate. The phosphate is transferred, first onto the imidazole side chain of a histidine residue in the enzyme and then onto GDP, producing GTP. Inosine diphosphate (IDP), another energy-phosphate acceptor, also functions as an alternate cosubstrate, in place of guanosine diphosphate. In that case, inosine triphosphate (ITP) is produced. This is a readily reversible reaction with $\Delta G^{\circ\prime}$ value –0.7 kcal/mol.

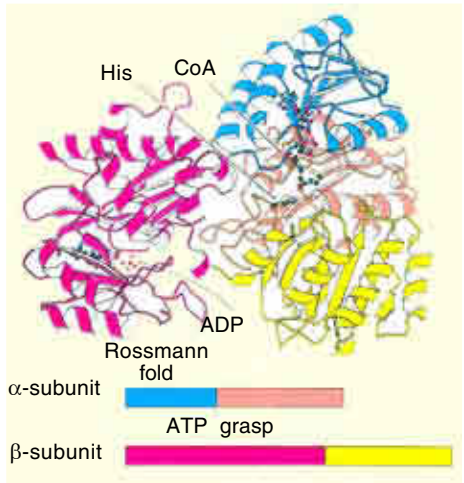
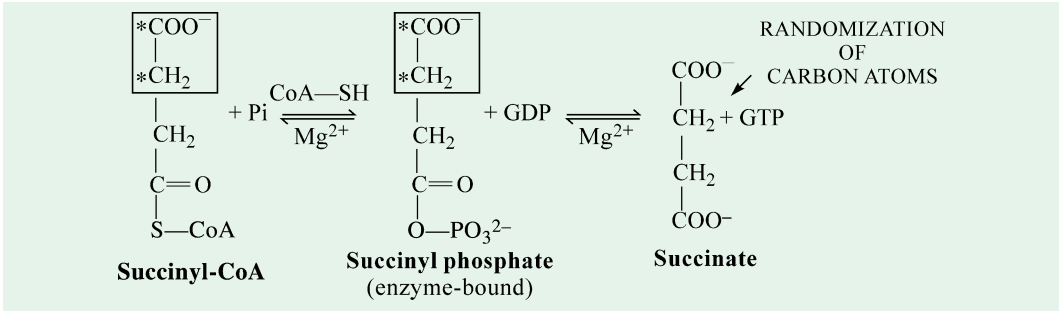


Fig. 22–10. Ribbon model of the structure of succinyl CoA synthetase
The enzyme is composed of two subunits. The α subunit contains a Rossmann fold that binds the ADP component of CoA, and the β subunit contains a nucleotide-activating region called the ATP-grasp domain. The ATP-grasp domain is shown here binding a molecule of ADP. The histidine residue picks up the phosphoryl group from near the CoA and swings over to transfer it to the nucleotide bound in the ATP-grasp domain.



The generation of a high-energy phosphate from succinyl-CoA is an example of a *substrate-level phosphorylation*. In fact, this is the only reaction in the citric acid cycle that directly yields a high-energy phosphate.

The GTP (or ITP) formed by succinyl-CoA synthase then readily donates its terminal phosphate group to ADP to form ATP by the action of Mg^{2+}-dependent enzyme, *nucleoside diphosphokinase* present in the interspace membrane of mitochondria. This is a reversible reaction ($\Delta G^{\circ\prime}$ = 0.0 kcal/mol).

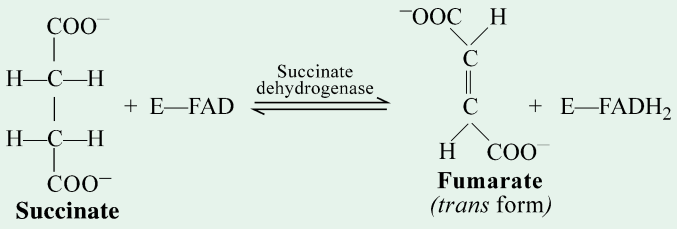
$$GTP + ADP \underset{Mg^{2+}}{\overset{\text{Nucleoside diphosphokinase}}{\rightleftharpoons}} GDP + ATP$$

$$ITP + ADP \underset{Mg^{2+}}{\overset{\text{Nucleoside diphosphokinase}}{\rightleftharpoons}} IDP + ATP$$

The remaining reaction steps of the citric acid cycle are concerned with the regeneration of oxaloacetate from succinate with a concomitant trapping of energy in the form of $FADH_2$ and NADH. Succinate is converted into oxaloacetate in 3 steps : an oxidation (Step 6), a hydration (Step 7) and a second oxidation (Step 8).

Step 6 : *Dehydrogenation of succinate to fumarate*

Succinic acid has the unique property of possessing two carbon atoms that are both α- and β-carbons (The same is true of certain other C_4 dicarboxylic acids). It is well known that such carbons are highly reactive and consequently would be expected to react rapidly under favourable conditions. The oxidation of succinate to fumarate is the only dehydrogenation in the citric acid cycle in which NAD^+ does not participate. Rather hydrogen is directly transferred from the substrate to flavoprotein enzyme. Here succinate, formed from succinyl-CoA, is dehydrogenated to fumarate (and not to its *cis*-isomer, maleate) by the flavoprotein enzyme, *succinate dehydrogenase*, located on the inner mitochondrial membrane. The enzyme contains the reducible prosthetic group flavin adenine dinucleotide (FAD) as the coenzyme. FAD functions as the hydrogen acceptor in this reaction, rather than NAD^+ (which is used in the other 3 oxidation- reductions in the cycle), because the free energy change is insufficient to reduce NAD^+. In succinate dehydrogenase, the isoalloxazine ring of FAD is covalently linked to a histidine side chain of the enzyme. The enzyme may, thus, be represented by E–FAD. Isotopic experiments have shown that *succinate dehydrogenase is specific for the trans hydrogen atoms of the 2 methylene carbons of succinate*, thus producing fumarate, which is in *trans* form. This points out to high geometrical specificity revealed by the enzyme. The reaction is readily reversible with a free energy change in the neighbourhood of 0.



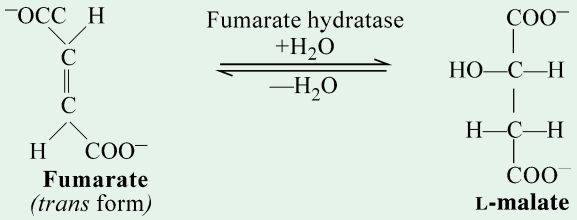
Succinate dehydrogenase from beef heart (MW = 97,000) consists of 2 subunits of unequal size. There is no heme in this enzyme. Rather, the iron atoms are bonded to the inorganic sulfide, resulting in the formation of iron-sulfur clusters or centres of varied molecular arrangement. The large subunit (MW = 70,000), which bears the active site, consists of FAD (rather than the riboflavin 5-phosphate) and two iron-sulfur clusters, each one of $(FeS)_2$ type whereas the small subunit (MW = 27,000) includes only a single iron-sulfur cluster of $(FeS)_4$ type. Electrons flow

from substrate to flavin through the nonheme iron groups on the large subunit to that on the small subunit. Succinate dehydrogenase differs from other enzymes in the citric acid cycle in that *it is an integral part of the inner mitochondrial membrane* and is tightly bound to it. In fact, the enzyme is directly linked to the electron-transport chain.

The $FADH_2$, produced by the oxidation of succinate, does not dissociate from the enzyme, in contrast with NADH. Rather two electrons from $NADH_2$ are transferred directly to the Fe^{3+} atoms of the enzyme. The enzyme transfers electrons directly to ubiquinone (formerly known as coenzyme Q) bypassing the first phosphorylation site in the electron transfer scheme. Thus, the overall transfer of electrons from succinate to oxygen results in the generation of only 2 high-energy phosphates. Addition of malonate or oxaloacetate inhibits succinate dehydrogenase competitively, resulting in accumulation of succinate.

Step 7 : *Hydration of fumarate to malate*

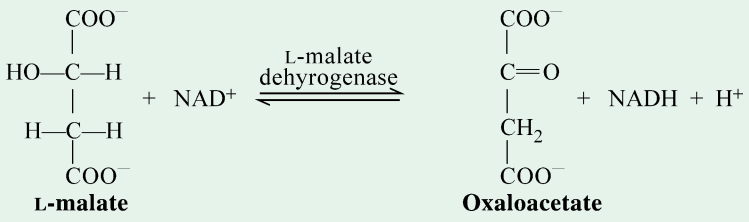
Fumarate is hydrated to form L-malate in the presence of *fumarate hydratase* (formerly known as *fumarase*). This is a freely reversible reaction ($\Delta G^{\circ\prime} \approx 0.0$ kcal/mol) and involves hydration in malate formation and dehydration in fumarate formation. *Fumarate hydratase is highly specific and catalyzes trans addition and removal of H and OH*, as shown by deuterium-labelling studies. The enzyme hydrates *trans* double bond of fumarate but does not act on maleate, the *cis*-isomer of fumarate. In the reverse direction, fumarase dehydrates L-malate and does not act on D-malate. Thus, there is absolute specificity for the *trans* decarboxylic unsaturated acid and the L-hydroxy dicarboxylic acid.



Fumarate hydratase from pig heart (MW = 2,20,000) is a tetramer of identical polypeptide subunits.The enzyme requires no cofactors but the participation of an acidic (*i.e.*, protonated) and a basic (*i.e.*, deprotonated) residue has been clearly implicated.

Step 8 : *Dehydrogenation of malate to oxaloacetate*

This is the fourth oxidation-reduction reaction in the citric acid cycle (the other 3 reactions being Steps 3, 4 and 6). Here L-malate is dehydrogenated to oxaloacetate in the presence of *l L-malate dehydrogenase*, which is present in the mitochondrial matrix. NAD^+, which remains linked to the enzyme molecule, acts as a hydrogen acceptor. This is a reversible reaction ($\Delta G^{\circ\prime} = +7.1$ kcal/mol). Although the equilibrium of this reaction is far to the left (*i.e.*, it favours malate formation), the reaction proceeds to the right since oxaloacetate and NADH, the two reaction products, are removed rapidly and continuously in further reactions. Oxaloacetate, so regenerated, allows repetition of the cycle and NADH participates in oxidative phosphorylation. This reaction, thus, completes the cycle.



L-malate dehydrogenase (MW = 66,000) consists of 2 subunits. It is a highly stereospecific NAD-requiring enzyme. Mammalian cells appear to contain 2 isozymes of malate dehydrogenase, one species probably being mitochondrial in localization. A high NAD^+/NADH ratio is stimulatory and a low ratio is inhibitory.

Fig 22-11 represents the coordinated control of glycolysis and the citric acid cycle by ATP, ADP, AMP and P.

STOICHIOMETRY OF THE CITRIC ACID CYCLE

A. Overall Balance Sheet

We have just seen that one turn of the citric acid cycle involves 8 enzyme-catalyzed reactions and leads to the conversion of one mole of acetyl-CoA to CO_2 plus H_2O. The net reaction of the cycle may be written as :

$$CH_3CO—SCoA + 3\ NAD^+ + FAD + GDP + Pi + 2\ H_2O$$

Acetyl–CoA

$$\xrightarrow{\text{Kerbs cycle}} 2CO_2 + CoA—SH + 3\ NADH + FADH_2 + GTP + 2\ H^+$$

Coenzyme A

The $\Delta G^{o\prime}$ for this overall reaction is –14.3 kcal/mol.

The net result of the Krebs cycle may be recapitulated as follows :

1. Two carbon atoms in the form of acetyl unit of acetyl-CoA enter the cycle and condense with oxaloacetate. Two carbon atoms emerge from the cycle as CO_2 in the two successive decarboxylation reactions (Steps 3 and 4) catalyzed by isocitrate dehydrogenase and α-ketoglutarate dehydrogenase respectively. It is noteworthy that the two C atoms that leave the cycle are different from the ones that entered in that round. However, additional turns around the cycle are required before the C atoms that entered as an acetyl group finally leave the cycle as CO_2.
2. Four pairs of hydrogen atoms are removed from the four cycle intermediates by enzymatic dehydrogenation (Steps 3, 4, 6 and 8). Three pairs of hydrogen are used to reduce 3 moles of NAD^+ to NADH and one pair to reduce the FAD of succinate dehydrogenase to $FADH_2$.
3. As would be clear in the folowing chapter, the four pairs of electrons, derived from the four pairs of hydrogen atoms removed in Steps 3, 4, 6 and 8, pass down the electron-transport chain and ultimately reduce 2 molecules of oxygen to form 4 molecules of water.

   $$3\ NADH + FADH_2 + 2\ O_2 \longrightarrow 3\ NAD^+ + FAD + 4\ H_2O$$

4. One high-energy phosphate bond (in the form of GTP) is generated from the energy-rich thioester linkage in succinyl-CoA (Step 5). GTP then donates its terminal phosphate group to ADP to produce a mole of ATP, which is thus a by-product of the cycle.
5. Two molecules of water are consumed : one in the synthesis of citrate (Step 1) and the other in the hydration of fumarate (Step 7).
6. *The citric acid cycle does not involve the net production or consumption of oxaloacetate (OAA) or of any other constituent of the cycle itself.* The cycle does not provide a route

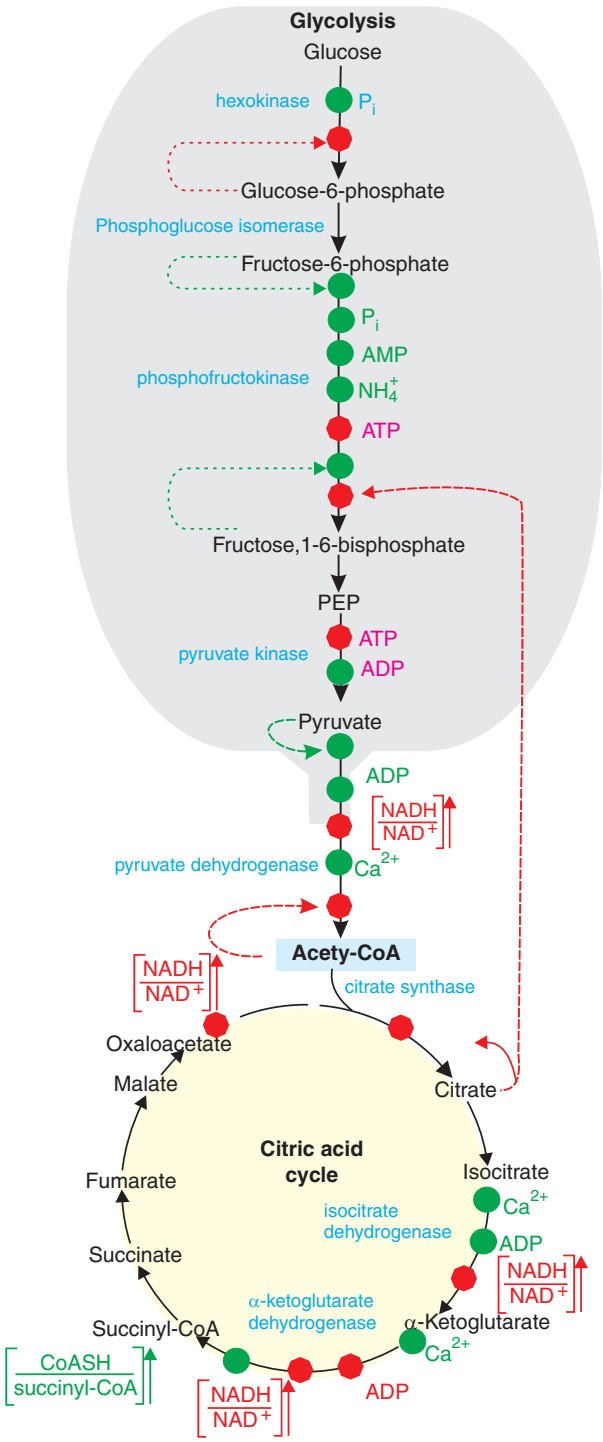


Fig. 22–11. A schematic of the coordinated control of glycolysis and the citric acid cycle by ATP, ADP, AMP, P_i, Ca^{2+}, and the [NADH]/[NAD^+] ratio

The vertical arrows indicate increases in this ratio. Here a green dot signifies activation and a red octagon represents inhibition.

for making additional OAA from acetyl groups. Failure to appreciate this point has led intelligent men into serious error in the past. However, at the end of each round, a mole of OAA is regenerated and the same condenses with the acetyl group of acetyl-CoA to continue the next round.

7. It is interesting to note that molecular oxygen deos not participate directly in the citric acid cycle. However, the cycle operates only under aerobic conditions because NAD^+ and FAD can be regenerated from their reduced forms (NADH and $FADH_2$) in the mitochondrion only by electron transfer to molecular oxygen. Thus, *whereas glycolysis has an aerobic and an anaerobic nature, the citric acid cycle is strictly aerobic in nature*.

B. Energy Yield

The main purpose of the citric acid cycle is not just the disposition of the carbon and hydrogens of all those compounds that can generate acetyl-CoA or any of the cycle members, but is *the conversion of potential chemical energy into metabolic energy in the form of ATP*. A grand total of 12 ATP molecules is formed during complete oxidation of one mole of acetyl-CoA, *i.e.*, during one turn of the citric acid cycle (refer Table 22–3). (However, a total of 15 ATP moles is formed when we start with the oxidation of 1 mole of pyruvate. This corresponds to a total of 38 ATP moles for the oxidation of 1 mole of glucose). Out of these 12 ATP molecules, only one ATP is produced directly at the level of the cycle itself (*i.e.*, at the substrate level) in the reaction catalyzed by succinyl-CoA synthase (Step 5). And the rest 11 ATP molecules are generated as a consequence of oxidation-reduction reactions of the cycle (Steps 3, 4, 6 and 8). Three molecules of NADH (one each in Steps 3, 4 and 8) and one mole of $FADH_2$ (Step 6) are produced in one turn of the cycle. The oxidation of one mole of NADH by O_2 results in the formation of 3 ATP molecules from ADP plus P_i, thus producing a total of 9 ATP molecules. However, the oxidation of $FADH_2$ by O_2 produces 2 molecules of ATP, rather than 3. Thus, a total of 12 ATP moles are generated utilizing 12 ADP and 12 P*i*.

Since 2 molecules of oxygen are consumed in the citric acid cycle, the P : O ratio (the ratio of high-energy phosphates to atoms of oxygen consumed) is 12/4 or 3.0. This value has a bearing on the relative energy production obtained from various fuels.

Table 22–3. Energy yield of the citric acid cycle

Step No.	*Reaction*	*Method of ATP Formation*	*ATP Yield Per Mole*
3	Isocitrate → α-ketoglutarate + CO_2	Respiratory chain oxidation of NADH	3
4	α-ketoglutarate → Succinyl-CoA + CO_2	Respiratory chain oxidation of NADH	3
5	Succinyl-CoA + ADP + P*i* → Succinate + ATP	Oxidation at substrate level	1
6	Succinate → Fumarate	Respiratory chain oxidation of $FADH_2$	2
8	Malate → Oxaloacetate	Respiratory chain oxidation of NADH	3
		Total gain of ATP = 12	

ROLE OF WATER IN THE CITRIC ACID CYCLE

Water is an extremely important metabolite for the various biologic processes. It functions as a solvent for the enzymes and most intermediates of the cycle. In addition, it also participates either as a reactant or as a product in many reactions of this path. *This ubiquitous character is nowhere more apparent than in the citric acid cycle.*

Water serves as a reactant in Steps 1 and 7. Moreover, it is both first eliminated and then utilized in Step 2 of the cycle. Furthermore, water is formed when the electron transport system (ETS) reoxidizes the NADH and FADH2, which are generated by the oxidoreduction reactions, to NAD^+ and FAD. Thus, water also appears as a product at 4 additional sites which are not directly the parts of the citric acid cycle. Finally, the elements of water are consumed when GTP is restored to GDP asnd P_i. Thus, *the citric acid cycle and the connected reactions consume 4 moles of water and release 5 moles.*

STEREOSPECIFICITY OF THE CITRIC ACID CYCLE

It has long been known that *aconitate hydratase* (catalyzing isomerization of citrate– Step 2), *succinate dehydrogenase* (catalyzing dehydrogenation of succinate– Step 6) and *fumarate hydratase* (catalyzing hydration of fumarate– Step 7) produce stereospecific products.

A. Biological Asymmetry of Citrate

Citrate (a C_6 compound) is a symmetric molecule but it behaves asymmetrically. This may be understood by tracing the fate of a particular carbon atom in the cycle. Suppose, in oxaloacetate (C_4), the carboxyl carbon furthest from the keto group is labelled with radioactive isotope of carbon *i.e.*, with ^{14}C. Analysis of ketoglutarate (C_5), formed later in the cycle, reveals that it contains the radioactive carbon and a mole of normal or nonradioactive CO_2, derived from the middle carboxylate group of citrate molecule, is given off along with it (Step 3). But succinate (C_4), formed at a latter stage in the cycle (Step 5), shows no radioactivity and all the radioactive carbon (^{14}C) has appeared in CO_2 released in the preceding reaction (*i.e.*, Step 4). This was quite a surprising result because citrate is a symmetric molecule and therefore it was assumed that the 2 —CH_2COO^- groups in the citrate mole would react identically. Thus, it was thought that if one citrate molecule reacts in a way as written above (refer Fig. 22.12, Path 1), another molecule of citrate would react in a way shown in Path 2, so that only half of the released CO_2 molecules should have labelled CO_2. But such an expectation did not come true. This peculiar behaviour of citrate can be explained on the basis of a **3-point attachment hypothesis** developed by Alexander G. Ogston (1948) of Oxford University. According to him, aconitase always acts on that part of citrate molecule which is derived from oxaloacetate (shown by boldface in Path 1). He further pointed out that the two carboxymethyl (—CH_2COO^-) groups of citrate are not truly geometrically equivalent and this nonequivalence becomes apparent upon its attachment on the enzyme surface by 3 points.

An illustration will make the point clear (Fig. 22–13). Consider a molecule which contains a centrally-located tetrahedral carbon atom. To this carbon atom are bonded 2H atoms (which are labelled as H_A and H_B), a group X and a different group Y. Suppose an enzyme binds 3 groups of this citrate, X, Y and H_A. It should, however, be noted that X, Y and H_B cannot be bound to this active site of the enzyme; two of these 3 groups can be bound but not all three. Thus, H_A and H_B are geometrically not equivalent and have different fates. Similarly, the two —CH_2COO^- groups in citrate molecule are geometrically not equivalent even though the citrate is optically inactive. This is due to the fact that the enzyme holds the substrate in a specific orientation.

An organic molecule, which has handedness and hence optically active, is called **chiral** molecule. Contrary

A compound is said to be **chiral** and to possess **chirality** if it cannot be superimposed on its mirror image, either as a result of simple reflection or after rotation and reflection. If superposition can be achieved, then the molecule is said to be **archiral**. The term chirality is equivalent to Pasteur's dissymetry.

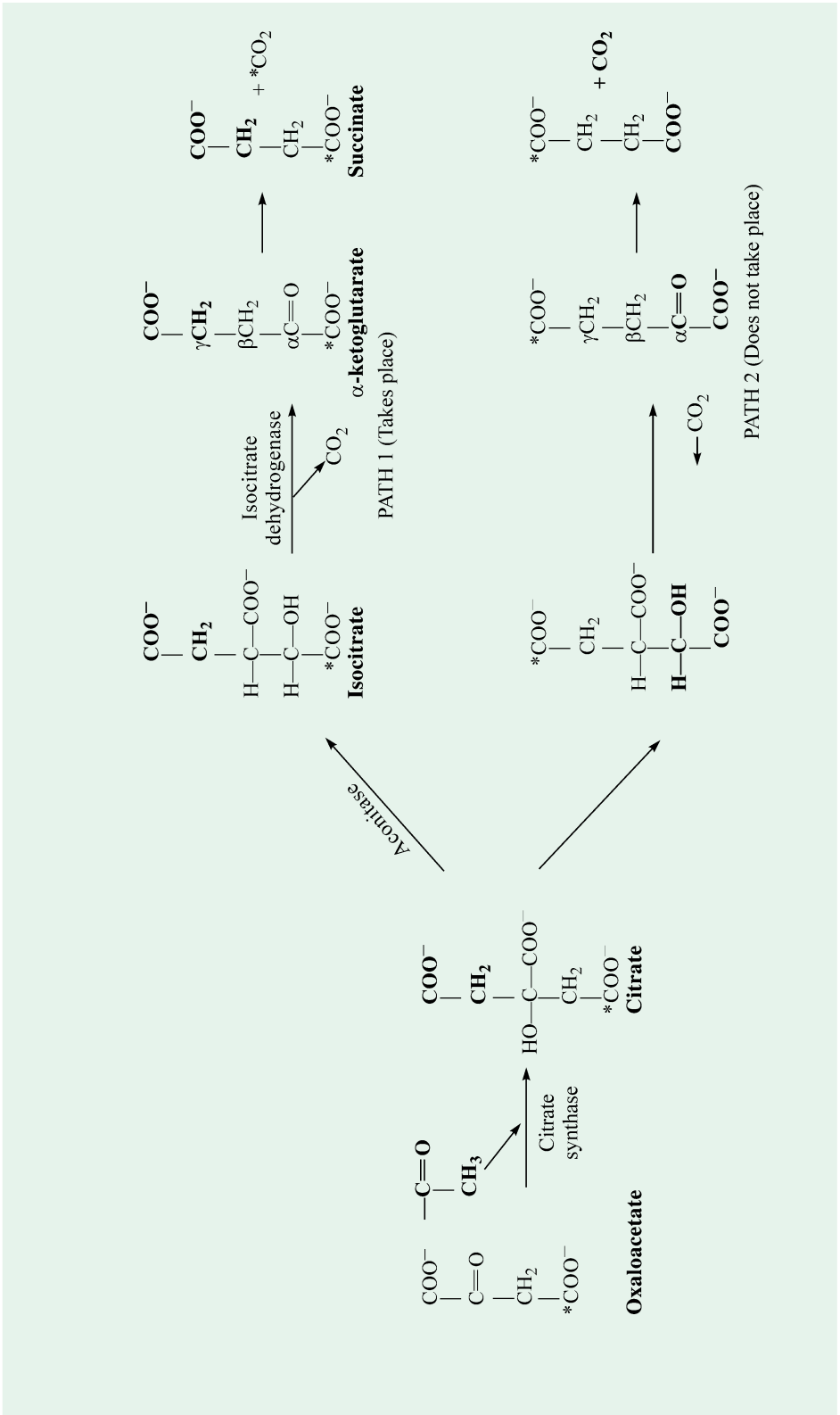


Fig. 22–12. Incorporation of isotopic carboxylate carbon (furthest from the keto group) of oxaloacetate into a-ketoglutarate

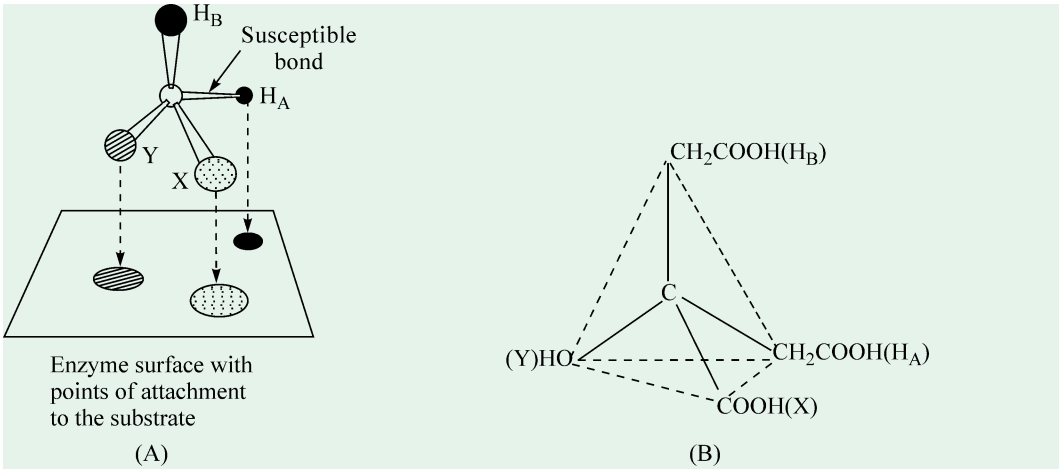


Fig. 22–13. Asymmetry of citrate and the action of aconitase
(A) Ogston's 3-point attachment hypothesis
(B) Structure of citrate

to this, a **prochiral** molecule, (such as citrate or CXY H_2) lacks handedness and is hence optically inactive. *The prochiral molecule can become chiral in one step*. For instance, CXY H_AH_B, which is a prochiral molecule, is transformed into a chiral one, CXYZ H_B, when one of its identical atoms or groups (H_A in this case) is replaced by another (Z in this case).

In citric acid cycle, one mole of acetyl-CoA is consumed and 2 moles of CO_2 are released. But neither of the carbon atoms lost as CO_2 is derived from that acetyl-CoA on the first turn of the cycle. However, at the level of free succinate, randomization does occur and the carboxyl carbon of acetate group of acetyl-CoA will be symmetrically distributed about the plane of symmetry. Hence, on the subsequent rounds of the cycle, with reappearance and reutilization of oxaloacetate, the acetyl carbon will be liberated as CO_2.

B. Geometrical Specificity of Succinate Dehydrogenase

It has already been discussed in Step 6 of the citric acid cycle.

C. Geometrical Specificity of Fumarate Hydratase

It has also been discussed in Step 7 of the same cycle.

REGULATION OF THE CITRIC ACID CYCLE

Since the citric acid cycle is one of the major routes of fuel consumption in most cells, there must be some control on the rate at which it proceeds. It would not do to have the cycle going full swing like a runaway boiler at times of rest nor would it do to have it sluggishly going when there is an immediate demand for ATP.

Several factors serve to control the rate of this sequence of reactions. These are described below:

1. Substrate Levels

One of the controlling features for any reaction sequence is the availability of the various substrates involved in it. It is known that the steady-state concentrations of the half-lives of most substrates of the cycle are of the order of a few seconds. *The outstanding exception is oxaloacetate (OAA), whose half-life is of the order of a tenth of a second.* This relatively restricted concentration of OAA puts it in high demand and also emphasizes its role in controlling the input of acetyl-CoA into the cycle. Regulation of the rate of this reaction would control activity in the enzyme cycle.

2. Enzyme Levels

All mitochondria, from widely different sources ranging from flight muscles of the locust to the various tissues of the rat, possess constant relative proportions of the various enzymes, including the characteristic dehydrogenases of the citric acid cycle. These observations suggest that there probably exists a genetic mechanism for the control of the synthesis or the integration of the key mitochondrial enzymes in the course of mitochondriogenesis. The genetic mechanism may involve a single operon containing all necessary structural genes to control enzyme biosynthesis. As a corollary, this genetic model requires other genes for the specification of related enzymes such as the cytoplasmic isocitrate dehydrogenases which occur in other metabolic systems.

3. Coenzyme Levels

As a general rule, catabolic energy-yielding processes generally require NAD^+, while anabolic energy-requiring ones almost invariably require $NADP^+$. These two coenzymes interact with one another according to the following equation.

$$NADH + NADP^+ \underset{}{\overset{\text{NAD(P) transhydrogenase}}{\rightleftharpoons}} NAD^+ + NADPH$$

It is apparent that such a system would represent a sensitive control point for the regulation of the levels of these coenzymes. The relative concentrations of NAD^+ and NADH are important in regulating metabolic pathways. When NAD^+/NADH ratio is high, the rate of citric acid cycle becomes rapid. However, the activity of this cycle is retarded when NAD^+/NADH ratio is low because of (*a*) insufficient NAD^+ concentration for otherwise normal enzymatic function, and (*b*) reoxidation of NADH coupled to ATP formation.

4. Respiratory Control

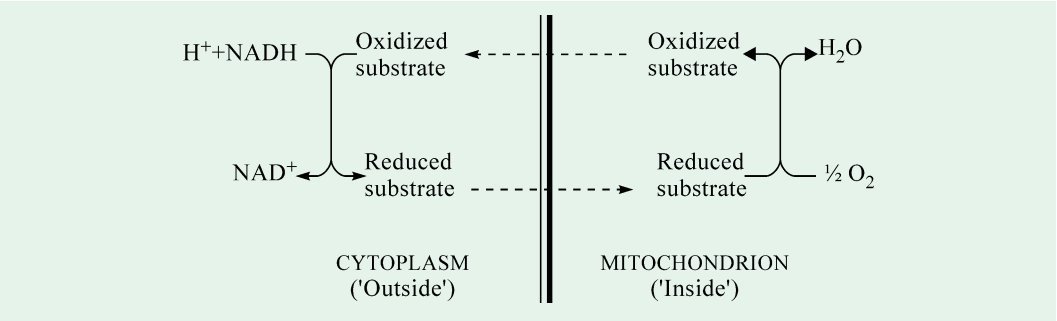
Respiration rate depends, not only on the nature and concentration of the substrates to be oxidized, but also on the coupling of respiration to phosphorylation. Intact mitochondria are usually 'tightly' coupled, so that their rate of respiration is actually controlled by the ratio [ADP]/[ATP]. When this ratio is high, respiration is promoted ("State 3"). In contrast, low ratios (*i.e.*, high ATP concentrations) decline respiration ("State 4"). Added ATP can even inhibit respiration because they bring about **reversed electron flow.** These phonomena are now known as respiratory control.

> The term **'reversed electron flow'** is used to describe certain oxidation-reduction reactions not ordinarily observed because of their unfavourable equilibrium which, however, can be 'reversed' by the addition of ATP.

5. Accessibility of Cycle Intermediates

The activity of the citric acid cycle is also controlled by its accessibility to acetyl-CoA of intermediates of the cycle. This problem consists of a combination of permeability barriers and geometry. The mitochondrial membrane itself provides a means for the admission of some substrates and the exclusion of others. A few examples are given below :

(*a*) Intact mitochondria do not allow NADH, which is produced on the 'outside' by oxidation of 3-phosphoglyceraldehyde during glycolysis, to enter 'inside' and with the result NADH is not connected directly to the electron transport system. Instead, NADH is reoxidized by a substrate. The reduced substrate then penetrates the mitochondrion and is reoxidized. The reduced substrate, thus, serves as a shuttle for electrons between NADH in cytoplasm and electron carriers in the motochondria.



(*b*) Similarly, acetyl-CoA generated in mitochondria does not readily diffuse out. Rather, it is first converted to citrate which is then cleaved in the cytoplasm to generate acetyl-CoA for various reactions taking place there.

(*c*) Another example of mitochondrial compartmentation is that of succinate dehydrogenase. Mitochondrial succinate dehydrogenase is freely available to succinate from outside the mitochondria but not to fumarate.

(*d*) Furthermore, added fumarate is also not freely accessible to the mitochondrial fumarate hydratase (= fumarase).

6. Ketosis

The accumulation of ketone bodies, acetoacetate and acetone formed by the liver in diabetics results from the production of more acetyl-CoA than can be cyclized via the Krebs cycle or other synthetic reactions. Under these conditions, the rate of Krebs cycle slows down probably due to hormonal action since ketone body formation (*i.e.*, ketosis) is affected by hormones of the hypophysis and adrenal cortex.

The CoA group of succinyl-CoA may be transferred to acetoacetate in mitochondria of muscle and kidney :

$$\text{Succinyl-CoA} + \text{Acetoacetate} \longrightarrow \text{Succinate} + \text{Acetoacetyl-CoA}$$

This is the point at which interaction between Krebs cycle and ketosis occurs, since acetoacetyl-CoA can be further degraded to acetyl-CoA.

7. Control of Enzyme Activity (= Regulation by Effectors)

Some of the reactions in the citric acid cycle require individual regulation (refer Fig. 22–14) because they are essentially irreversible under physiological conditions. There is always a danger from such reactions because they may continue till they have consumed the available supply of substrate or coenzyme. In general, at many points, stimulation or inhibition of the cycle is determined by the relative levels of NADH/NAD^+, ATP/ADP, acetyl-CoA/CoA or succinyl-CoA/CoA. When these ratios are high, the cell has ample supply of energy and flow through the cycle is slowed. When these ratios are low, the cell is in need of energy and flow through the cycle quickens.

(*a*) **Citrate synthase reaction.** As in most metabolic cycles, the initial steps of the citric acid cycle are believed to be rate-setting for the cycle as a whole. The rate of citrate synthesis from oxaloacetate and acetyl-CoA is controlled by the concentration of acetyl-CoA itself which is, in turn, governed by the activity of pyruvate dehydrogenase complex. The concentration of oxaloacetate is also most important factor since its concentration in mitochondria is very low and depends upon metabolic conditions. ATP is an allosteric inhibitor of citrate synthase. The effect of ATP is to increase the K_m for acetyl-CoA. Thus, as the level of ATP increases, the affinity of the enzyme towards its substrate (acetyl-CoA) decreases and so less citrate is formed. The activity of citrate synthase is also regulated

by the concentration of succinyl-CoA, a later intermediate of the cycle. High succinyl-CoA levels also decrease the affinity of citrate synthase towards acetyl-CoA. Furthermore, the citrate is a competitive inhibitor for oxaloacetate on the enzyme. The effect is *double-barrelled*. An accumulation of citrate raises its concentration as an inhibitor, but it also lowers the concentration of oxaloacetate (OAA) as a substrate. This is because the complete cycle must function at the same rate to restore the OAA consumed in the first step. *Any accumulation of intermediates in the cycle represents a depletion of oxaloacetate.*

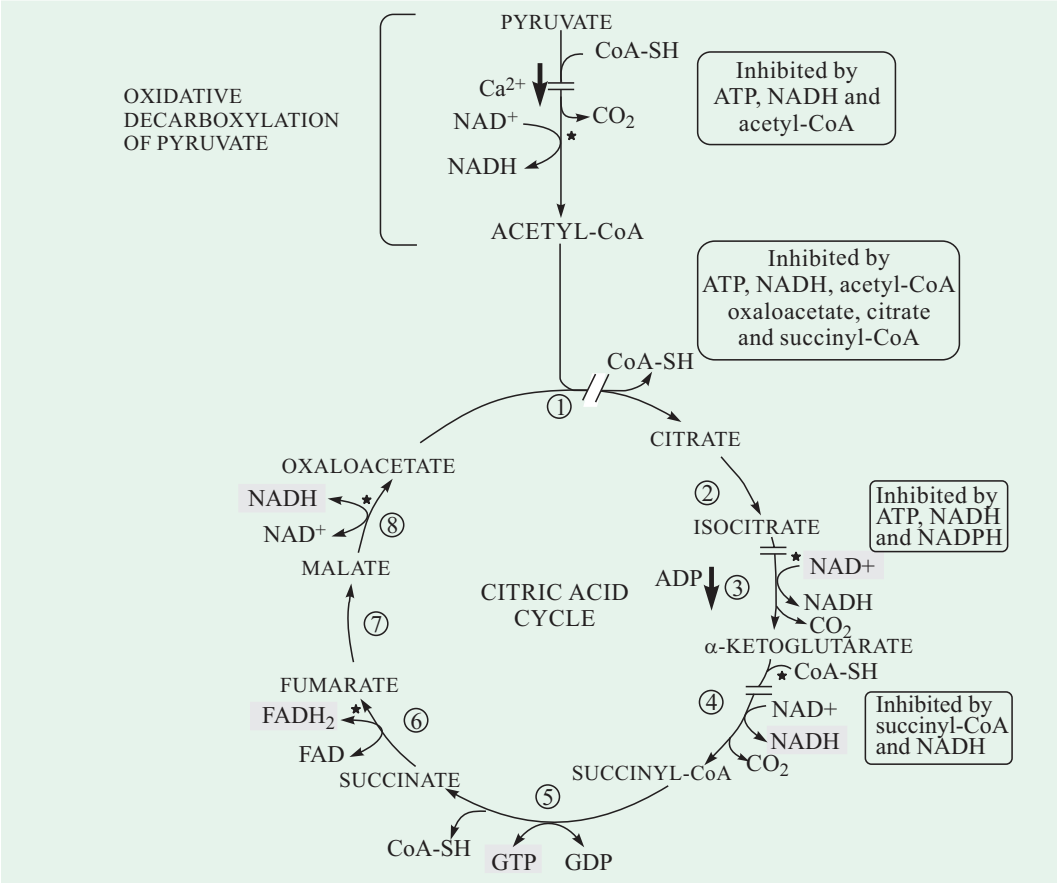


Fig. 22–14. Control of the oxidative decarboxylation of pyruvate and the citric acid cycle

Circled numbers correspond to the reaction steps of the citric acid cycle. Thick arrows indicate the stimulatory effect of the molecule or ion mentioned. Starred reactions indicate steps that require an electron acceptor (NAD^+ or FAD) that is regenerated by the respiratory chain.

(*b*) **Isocitrate dehydrogenase reaction.** *This reaction appears to be the rate-limiting step of the citric acid cycle.* Mammalian isocitrate dehydrogenase is allosterically stimulated by ADP which enhances its activity for the substrate whereas NADPH is an allosteric inhibitor. NADH and ATP are the competitive inhibitors for the NAD^+ site. NADH and NADPH bind to different sites on the enzymes. However, in the the case of yeast and *Neurospora*, AMP rather than ADP is the main stimulator (or positive effector) of the enzyme.

(*c*) **α-ketoglutarate dehydrogenase reaction.** The activity of a-ketoglutarate dehydrogenase complex is inhibited by its two products, succinyl-CoA and NADH, the former being more effective. In fact, succinyl-CoA is a competitive inhibitor for one of its substrate, coenzyme A. Here again is a *double-barrelled effect*. A rise in succinyl-CoA concentration in itself inhibits but it also represents a depletion of coenzyme A, leading to still more

Table 22–4. Some controls on the citric acid cycle and connected systems

Enzyme system	*Location*	*Reactant(s)*	*Products(s)*	*Activator(s)*	*Inhibitor(s)*	*Remarks*
1. ETS-Oxidative phosphorylation	Microconidia	ATP, P_i	ATP, CO_2 (by virture of respiration)	—	ATP uncouplers	Coupled ATP production
2. Pyruate carboxylase	Mitochondria	ATP, O_2	ADP	Acetyl-CoA	—	Controls carbohydrate synthesis
3. Acetyl-CoA carboxylase	Cytoplasm	ATP, CO_2	ADP	Citrate etc	Long-chain acyl-CoAs	Controls fatty acid synthesis
4. Citrate synthase (= condensing enzyme)	Mitochondria	Acetyl-CoA, Oxaloacetate	Citrate, CoA—SH	—	Long-chain acyl-CoAs ATP or NADH*	Controls diversion of acetyl-CoA to other pathways
5. Citrate lyase (ATP-requiring)	Cytoplasm	Citrate (from acetyl-CoA)	Acetyl-CoA, Oxaloacetate	—	—	Makes available extramitochondiral acetyl-CoA for lipid synthesis
6. Isocitrate lyase	Cytoplasm	Citrate (from acetyl-CoA)	Succinate	—	Phosphoenol-pyruate	Controls combination of C_2 units (in bacterial and plants only)
7A. Isocitrate dehydrogenase (NAD-specific)	Mitochondria	NAD^+	NADH, CO_2	ADP	ATP, NADH	Oxidizes isocitrate
7B. Isocitrate dehydrogenase (NADP-specific)	Cytoplasm Mitochondria	$NADP^+$	NADPH, CO_2	Oxaloacetate ?	—	Generates NADPH
8. Glutamate dehydrogenase	Mitochondria	NADH or NADPH, NH_3	NAD^+ or $NADP^+$	ADP	GDP + NADH	Precise nature of control not known

* Mammalian and yeast enzymes are inhibited by ATP whereas *Escherichia coli* enzyme is inhibited by NADH. Both inhibition controls are of the feedback type.

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effective inhibition. *The α-ketoglutarate dehydrogenase reaction represents a threat to the supply of coenzyme A which is needed for other reactions.* Indeed 70% of the coenzyme A supply in some tissues is present as succinyl-CoA under some conditions, even though the enzyme is regulated.

The various controls of the citric acid cycle have been listed in Table 22–4.

AMPHIBOLIC ROLES OF THE CITRIC ACID CYCLE

The citric acid cycle has a dual or amphibolic (*amphi*^{G} = both) nature. The cycle functions not in the oxidative catabolism of carbohydrates, fatty acids and amino acids but also as the first stage in many biosynthetic (= anabolic) pathways, for which it provides precursors. Under certain conditions, one or more intermediates of the cycle may be drawn off for synthesis of other metabolites. In order to avoid cutting the cycle, the intermediates of the cycle are, however, replenished at the same or different locus by other reactions called as anaplerotic reactions. Under normal conditions, the reactions by which the cycle intermediates are drained away and those by which they are replenished are in dynamic balance so that at any time the concentration of the citric acid cycle intermediates in mitochondria usually remains relatively constant. Thus, for example, if a cell over a period of time needs to make 4.25 m moles of glutamic acid from α-ketoglutarate, it must provide 4.25 μ moles of acetyl-CoA and 4.25 μ moles of oxaloacetate (the two compounds from which synthesis will occur) to "balance the books", so to speak. The biosynthetic and anaplerotic functions of the cycle (refer Fig. 22–15) are discussed below.

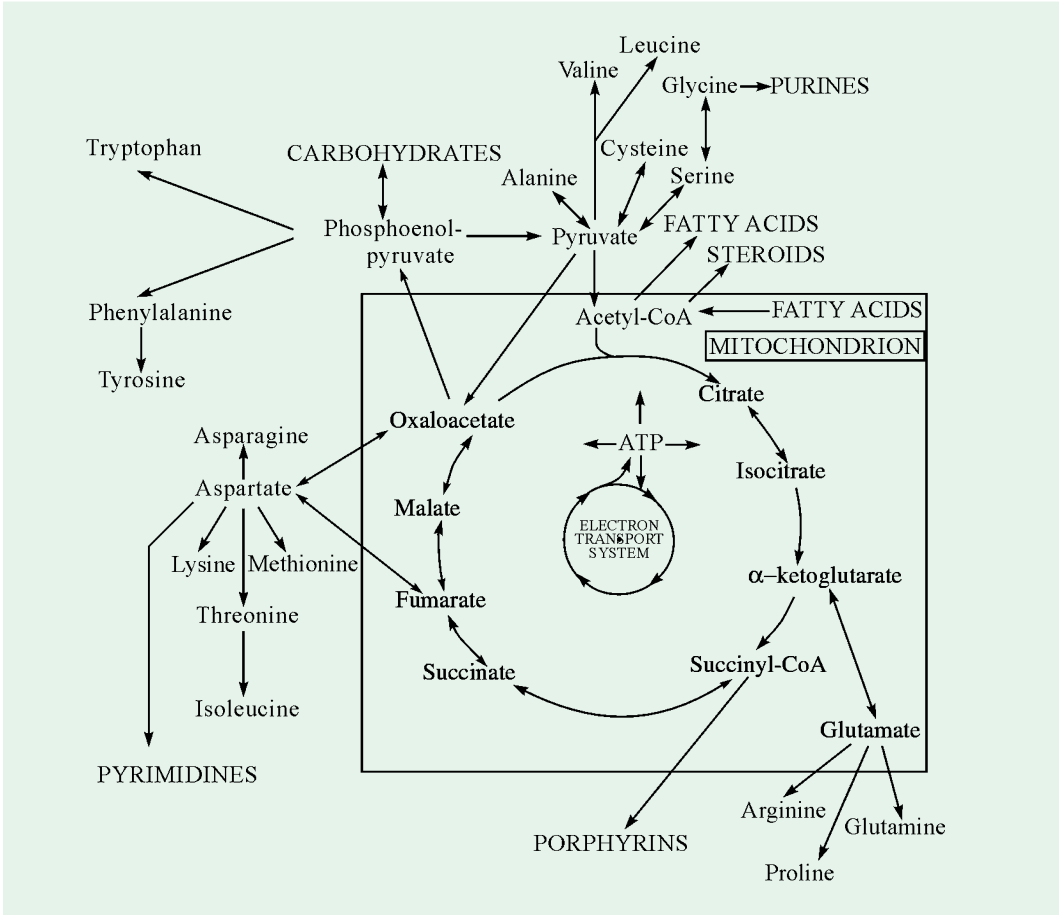


Fig. 22–15. Biosynthetic and anaplerotic functions of the citric acid cycle

A. Biosynthetic Roles

Citric acid cycle is the primary source of some key metabolites of the cell as it provides intermediates for their biosyntheses. In fact, intermediates from the cycle serve as biosynthetic precursors of all 4 major classes of compounds *viz*., carbohydrates, lipids, proteins and nucleic acids.

1. Carbohydrates. The intermediates of the citric acid cycle are, as such, probably not important in the biosynthesis of carbohydrates by most animal cells. However, when such cells have ample supply of C_4 acids or C_3 compounds, certain mitochondrial reactions involving oxaloacetate become important routes to carbohydrate synthesis. *The general process appears to be the conversion of such C_3 or C_4 compounds to oxaloacetate.* For example, if sufficient succinate is available, it passes through a portion of cycle to oxaloacetate. However, other compounds can be first converted to pyruvate. The pyruvate is, then, carboxylated to oxaloacetate in the presence of pyruvate carboxylase to which biotin is covalently attached (The reaction is discussed at length in the following subheading). Although, *Escherichia coli* might convert pyruvate to phosphoenolpyruvate (PEP) with a single ATP per unit, it appears that the animals require the equivalent of two ATP's. The carbohydrate is, then, obtained from PEP by reversal of the EMP pathway.

2. Lipids. Acetyl-CoA is the key intermediate in the biosynthesis of lipids. It is generated by just 2 main processes :

(*a*) the thiolytic cleavage of acetoacetyl-CoA, which is generated by the oxidation of fatty acids and certain amino acids.

(*b*) the oxidative decarboxylation of pyruvate.

Yet the biosynthesis of fatty acids requires an obligatory first step, the carboxylation of acetyl-CoA to malonyl-CoA which is probably catalyzed by an extramitochondrial enzyme complex. Acetyl-CoA is first converted to citrate within the mitochondrion by condensation with oxaloacetate. The citrate, so formed, then passes out to the cytoplasm where it is cleaved back to oxaloacetate plus acetyl-CoA by the ATP-requiring enzyme, citrate lyase which is also known as citarate cleavage enzyme.

$$\text{Citrate} + \text{ATP} + \text{CoA—SH} + \text{Enz} \underset{\text{Condensation}}{\overset{\text{Citrate lyase}}{\rightleftharpoons}} [\text{Enz-citryl~SCoA}] + \text{ADP} + \text{P}i$$

$$[\text{Enz—citryl~SCoA}] \xrightarrow[\text{Hydrolysis}]{+\ H_2O} \text{Acetyl-CoA} + \text{OAA} + \text{Enz}$$

This reaction, unlike that responsible for the synthesis of carbohydrates, requires only catalytic amounts of OAA and synthesis of fatty acids, therefore, does not require the obligatory participation of C_4 dicarboxylic acids.

Fatty acids obtained by the hydrolysis of lipids are catabolized in the mitochondrion and contribute acetyl-CoA for its breakdown by the citric acid cycle. Acetyl-CoA, from this source or synthesized by the PDC system in the outer membrane of the mitochondrion, is transferred outside for the biosynthesis of fatty acids and steroids.

3. Proteins. Protein synthesis, like all the other synthetic processes, requires a supply of monomeric units or precursors. In the case of proteins, the monomeric units are some 20 L-amino acids. Most higher animals are unable to synthesize about half of these amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val) in sufficient amounts to meet their demands. Plants and most microorganisms, on the contrary, are able to synthesize almost all the amino acids. Again, the citric acid cycle provides a means for the generation of 'nonessential' amino acids in animals and the bulk of all amino acids in other cells and organisms.

Two of these amino acids (glutamate and aspartate) are obtained directly from intermediates of the citric acid cycle and one of these (alanine) is made directly from pyruvate. Glutamate is made directly from a-ketoglutarate by all cells. Aspartate is made by most cells from oxaloacetate.

Bacterial cells have got the additional capacity of producing aspartate from fumarate, although animals cannot. All other amino acids are made indirectly from glutamate, aspartate and alanine by series of reactions. Thus, there exist 3 'families' of amino acids that originate from the 3 -keto acids, namely a-ketoglutarate, oxaloacetate and pyruvate. These are :

(*a*) **Glutamic Family** — This group consists of glutamate, glutamine, arginine and proline. These amino acids are formed throughout the living organisms.

(*b*) **Aspartic Family** — This group includes aspartate, asparagine, lysine, methionine, threonine and isoleucine. These are simply not produced at all by mammals and perhaps not by other animals.

(*c*) **Pyruvic Family** — This group comprises of alanine, valine, leucine, cysteine, serine, glycine, histidine, tryptophan, phenylalanine and tyrosine. Only some of these are synthesized in higher organisms.

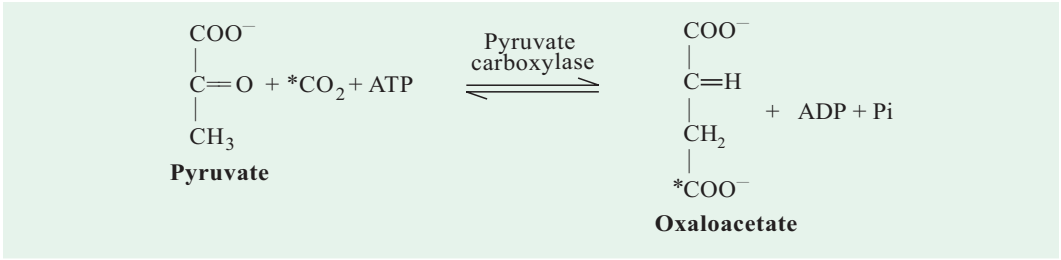
4. Purines and pyrimidines. Purines and pyrimidines are important constituents of the coenzymes and nucleic acids. Aspartate provides the carbon skeleton of pyrimidines whereas glycine from pyruvate contributes the carbon skeleton for purines.

5. Porphyrins. Porphyrins are essential components of the respiratory pigments and enzymes. Animals utilize succinyl-CoA as one of the components for the synthesis of porphyrins.

B. Anaplerotic Roles

Many intermediates of the citric acid cycle are used for the synthesis of other substances (as seen in the preceding subsection). The drain of these intermediates would ultimately prevent operation of the cycle were they not replenished. H. L. Kornberg (1966) has proposed the term **anaplerotic** for these replenishing or "filling-up" reactions. *Anaplerosis is defined as any reaction that can restore the concentration of a crucial but depleted intermediate.* Some important anaplerotic reactions of the citric acid cycle are listed below :

1. Pyruvate carboxylase reaction. The citric acid cycle will cease to operate unless oxaloacetate is formed *de novo* because acetyl-CoA cannot enter the cycle unless it condenses with oxaloacetate. Mammals lack the enzyme system needed to convert acetyl-CoA into oxaloacetate or another citric acid cycle intermediate. However, in animal tissues *esp*., liver and kidney, oxaloacetate is formed by the carboxylation of pyruvate through the action of *pyruvate carboxylase*, a mitochondrial complex regulatory enzyme. Since the standard free-energy change of the reaction is very small ($\Delta G^{o\prime}$ = 0.5 kcal/mole), it is a readily reversible reaction.



[the fate of CO_2-carbon is shown with an asterisk in this and subsequent reactions]

Pyruvate carboxylase (MW, of that from chicken liver = 6,60,000) is a tetramer of 4 identical subunits. Each subunit contains an active site, including tightly-bound Mn^{2+} (or Mg^{2+}) and covalently-bound biotin, and also an allosteric site, which binds acetyl-CoA. Biotin is covalently attached through an amide linkage with the $\in$-NH_2 group of a specific lysine residue in the active site. Pyruvate kinase has an unusual metal requirement : a monovalent cation (K^+) and a divalent cation (Mg^{2+} or Mn^{2+}). The enzyme has nearly absolute requirement for acetyl-coenzyme A as an activator.

The carboxylation of pyruvate occurs in 2 steps, each of which is catalyzed by a distinct subsite of the active site :

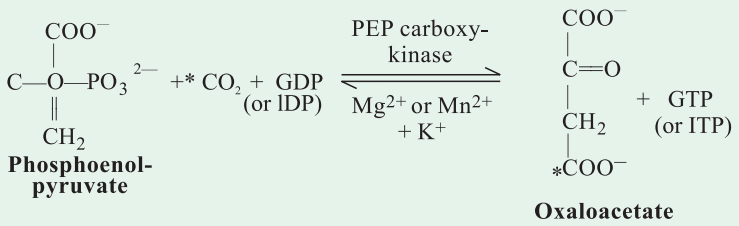
(*a*) **First step :** Free CO_2, the precursor of new carboxyl group of oxaloacetate, is first, 'energized' through its covalent union with a ring N atom of biotin, forming 1′-N-carboxybiotinyl enzyme. The energy needed is provided by ATP. The rate of this reaction is accelerated by acetyl-CoA which acts as an allosteric modulator.

$$ATP + CO_2 + \text{Enz-biotin} + H_2O \rightleftharpoons ADP + P_i + \text{Enz-biotin-}COO^- + 2H^+$$

(*b*) **Second step :** The new carboxyl group covalently bound to the biotin prosthetic group is transferred to pyruvate to form oxaloacetate.

$$\text{Enz-biotin-}COO^- + \text{Pyruvate} \rightleftharpoons \text{Enz-biotin} + \text{Oxaloacetate}$$

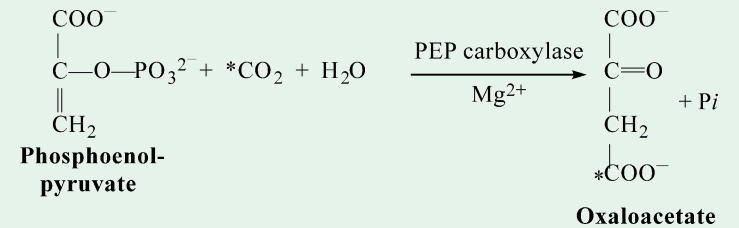
2. Phosphoenolpyruvate carboxykinase reaction. This is another anaplerotic reaction that feeds the Krebs cycle and occurs in animal tissues, *esp*., heart and muscles. This reversible reaction brings about carboxylation of phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) with the concomitant use of GDP (or IDP) as phosphate acceptor, by the enzyme *phosphoenolpyruvate carboxykinase* (= PEP *carboxykinase*). The breakdown of phosphoenolpyruvate, a super-energy compound, furnishes the energy for the carboxylation to yield oxaloacetate and also for the phosphorylation of GDP to yield GTP. However, the affinity of the enzyme for oxaloacetate is very high ($K_m = 2 \times 10^{-6}$) while that for CO_2 is low. Hence, the enzyme favours PEP formation.



Phosphoenol-pyruvate — **Oxaloacetate**

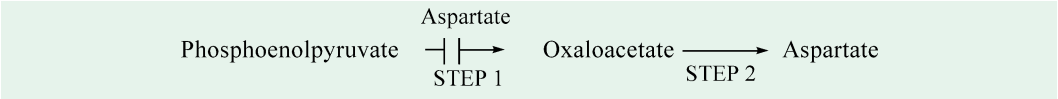
PEP carboxykinase (MW = 75,000) is found primarily in cytosol and to less extent in mitochondria. It differs in mechanism from that of pyruvate carboxylase as it does not involve biotin and also CO_2 is not 'activated'.

3. Phosphoenolpyruvate carboxylase reaction. This is another replenishing reaction of the citric acid cycle and occurs chiefly in higher plants, yeast and bacteria (except pseudomonads) but not in animals. In this reaction, phosphoenolpyruvate (PEP) is irreversibly carboxylated to oxaloacetate *by phosphoenolpyruvate carboxylase (= PEP carboxylase).*

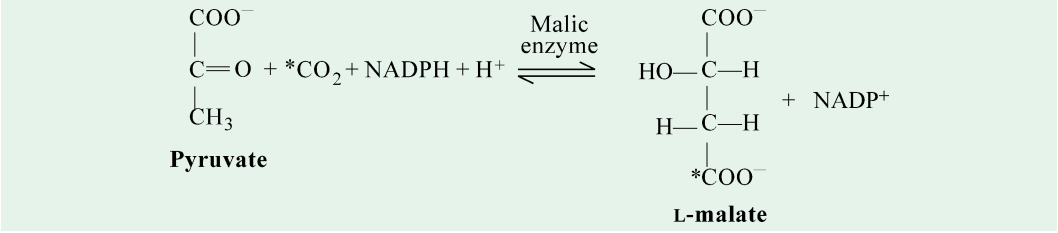


Phosphoenol-pyruvate — **Oxaloacetate**

PEP carboxylase has the same function as that of pyruvate carboxylase, *i.e*., to ensure that the citric acid cycle has an adequate supply of oxaloacetate. The enzyme requires Mg^{2+} for activity. PEP carboxylase is activated by fructose 1, 6-diphosphate and inhibited by aspartate. The inhibitory effect is understandable because oxaloacetate is the direct precursor of aspartate by transamination. The above biosynthetic sequence is a simple means for synthesizing aspartate from PEP, and aspartate can control its own formation by inhibiting the Ist step of this sequence.



4. Malic enzyme reaction. The reaction occurs in plants, in several animal tissues and in some bacteria grown on malic acid. Malic enzyme catalyzes the reversible formation of L-malate from pyruvate and CO_2



Malic enzyme is found in two different forms, one in cytosol and another in mitochondria. Its major function is probably the formation of NADPH required for biosynthetic processes, or the reverse of the reaction as written. Mitochondrial malic enzyme, but not the cytosolic form, is an allosteric enzyme. Succinate is a positive effector (*i.e.*, activator) and decreases the K_m for malate.

Carbon Dioxide-fixation Reactions (= Wood-Werkmann's Reactions)

Wood and Werkmann (1936) observed that when propionic acid bacteria fermented glycerol to propionic and succinic acids, more carbon was found in the products than the reactant, glycerol. Moreover, carbon dioxide proved to be the source of the extra carbon atoms or the carbon that was 'fixed'. Today, the physiological significance of CO_2-fixation includes not only the metabolism of propionic acid bacteria but also the anaplerotic reactions catalyzed by acetyl-CoA carboxylase, propionyl-CoA carboxylase etc.

MODIFICATION OF THE CITRIC ACID CYCLE : GLYOXYLATE CYCLE

Higher plants and some microorganisms (bacteria, yeast and molds), under certain specific conditions, face the problem of converting fats into 2-carbon compounds (such as acetyl-CoA) into carbohydrates and other cell constituents *via* the fundamental pathway of Krebs cycle. Such conditions occur in higher plants during germination of their seeds which contain large quantities of stored lipid and in microorganisms when they are grown on ethanol or acetate which function as the sole source of carbon in them. The task (of converting fats into carbohydrates) in these organisms is, however, accomplished by means of a cyclic set of reactions called **glyoxylate cycle** or **Krebs–Kornberg cycle**, the latter nomenclature based on its two principal investigators, Hans A. Krebs and H. L. Kornberg, both of whom discovered this cycle in 1957. The glyoxylate cycle (refer Fig. 22–16) utilizes five enzymes, of which two namely *isocitrate lyase and malate synthase* are absent in animals. Hence, *the glyoxylate pathway does not occur in animals.* However, plant cells contain all the 5 enzymes required for the cycle, in subcellular organelles called *glyoxysomes*, hence this cycle is operative in them. It is interesting to note that the glyoxysomes appear in the cotyledons of lipid-rich seeds (*e.g.*, groundnut, castor, bean) shortly after germination begins and at a time when lipids are being utilized as the major source of carbon for carbohydrate synthesis.

In effect, the glyoxylate cycle bypasses steps 3 to 7 of the citric acid cycle, thereby omitting the two oxidative decarboxylation reactions (Steps 3 and 4) in which CO_2 is produced. The bypass consists of 2 reactions, namely :

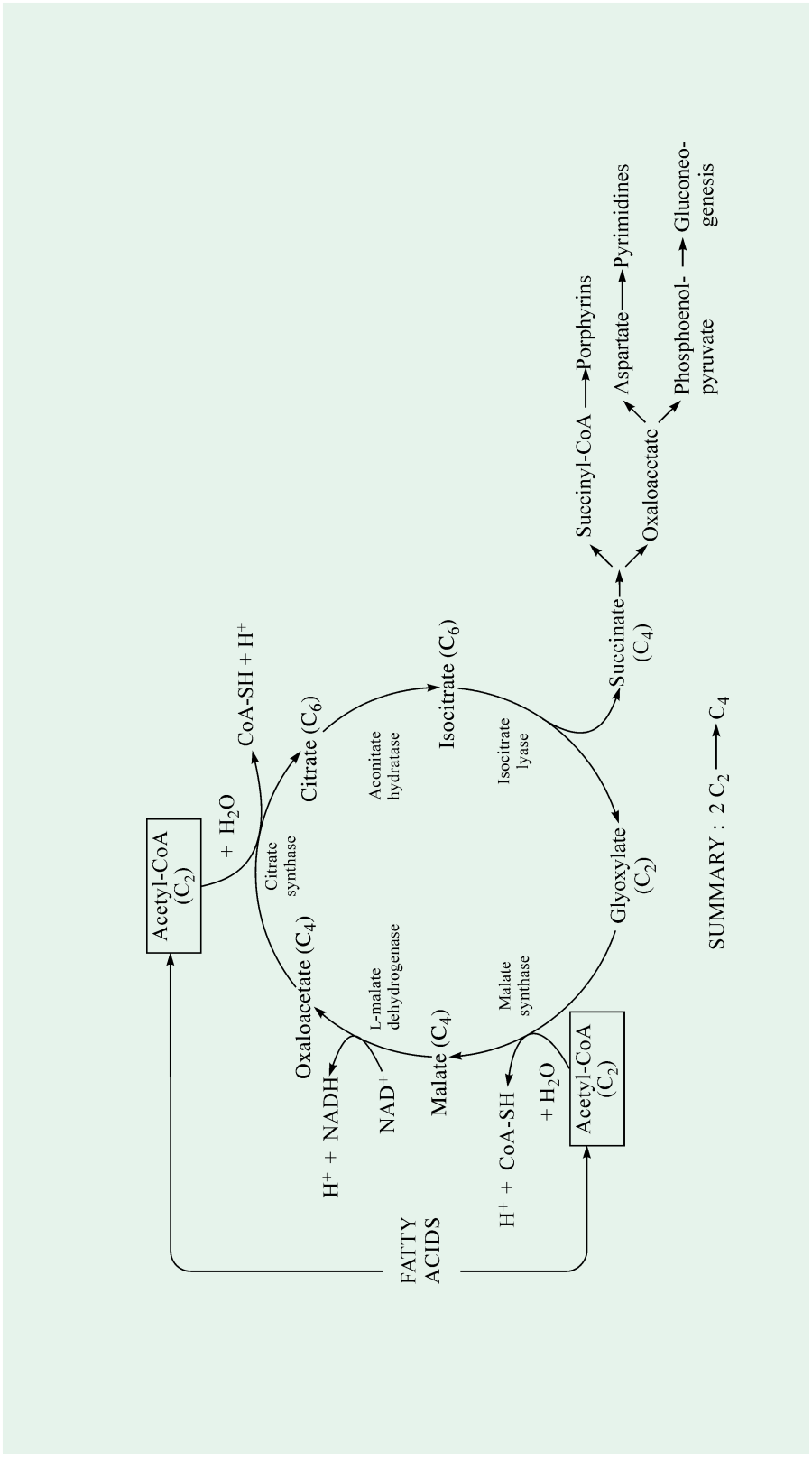


Fig. 22–16. The glyoxylate cycle

Oxidation of fatty acids can provide acetyl-CoA, which enters the cycle at two points. Each turn of the cycle results in generation of one mole of succinate from 2 moles of acetyl-CoA. The net result of the bypass is the formation of C–C bonds between C_2 subunits.

(*a*) splitting of isocitrate into succinate and glyoxylate, and

(*b*) conversion of glyoxylate into malate.

Thus, the glyoxylate cycle consists of 5 enzyme-catalyzed steps (Fig. 22–8), of which 3 steps are the same as in Krebs cycle, *viz*., Steps 1, 2 and 8. The various steps are sequentially described as follows :

Step 1 : *Condensation of acetyl-CoA with oxaloacetate*

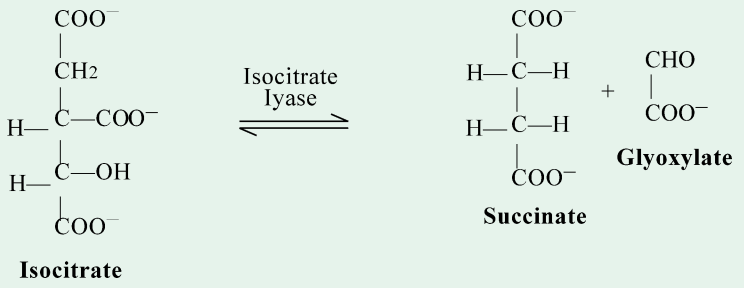
This reaction is catalyzed by the enzyme, *citrate synthase* (For details, see page 394).

Step 2 : *Isomerization of citrate into isocitrate*

This reaction is catalyzed by the enzyme, *aconitate hydratase* which is also known as *aconitase* (For details, see page 395).

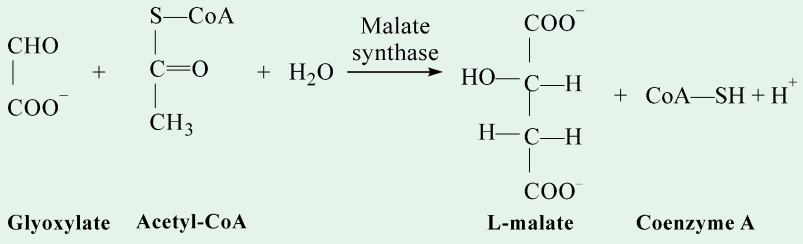
Step 3 : *Cleavage of isocitrate into succinate and glyoxylate*

Isocitrate, instead of being oxidized *via* the CAC, is rather split into succinate and *glyoxylate* by the enzyme, *isocitrate lyase* (= *isocitrase* or *isocitratase*). The enzyme is, thus, present at the branch point of two metabolic pathways (*i.e*., citric acid cycle and glyoxylate cycle) and appears to be susceptible to second-site control. Isocitrate lyase from *Escherichia coli* is, however, inhibited by phosphoenolpyruvate.



Step 4 : ***Conversion of glyoxylate into malate***

The glyoxylate, so formed, then condenses with a mole of acetyl-CoA to produce L-malic acid by the enzyme, *malate synthase*. This reaction is analogous to that of citrate synthase reaction (Step 1) of the citric acid cycle.



Step 5 : *Dehydrogenation of malate to oxaloacetate*

L-malate is then dehydrogenated, in the presence of *L-malate dehydrogenase*, to oxaloacetate (For details, see page 401). The oxaloacetate, so formed, can condense with another mole of acetyl-CoA to start another turn of the cycle. It may, however, be pointed out that *whereas in the citric acid cycle the conversion of isocitrate to malate is an aerobic process, in glyxylate cycle the conversion takes place anaerobically.*

To summarize, operation of glyoxylate cycle, in addition to the 2 novel reactions, also requires the participation of 3 of the enzymes of the citric acid cycle, *viz*., citrate synthase, aconitate hydratase and malate dehydrogenase. Also required is the electron transport chain for the oxidation of NADH (produced in Step 5) by molecular oxygen. This plus the malate synthase reaction (Step 4) provide the essential driving force for the cycle. One turn of the cycle leads to the oxidation of 2 moles of acetyl-CoA to yield 1 mole of succinate with the simultaneous removal of two reducing equivalents. The overall reaction for the glyoxylate cycle may be written as :

$$\underset{\text{Acetyl-CoA}}{2\ CH_3CO{-}S{-}CoA} + NAD^+ + 2\ H_2O \xrightarrow{\textbf{Glyoxylate cycle}} \underset{\text{Succinate}}{\begin{matrix}CH_2{-}COO^- \\ | \\ CH_2{-}COO^-\end{matrix}} + \underset{\text{Coenzyme A}}{2\ CoA{-}SH} + NADH + 3\ H^+$$

The succinate, so formed, may be used for biosynthetic purposes. For example,

(*a*) Succinate can be converted to succinyl-CoA (Step 5 of the citric acid cycle) and serve as a precursor of porphyrins.

(*b*) Succinate can be oxidized to oxaloacetate (*via* Steps 6, 7 and 8 of the citric acid cycle) which can be utilized for the synthesis of aspartate and other compounds (such as pyrimidines), derived from aspartate.

(*c*) Oxaloacetate can also be converted into phosphoenolpyruvate (For details, see page 413) which is then used for the reactions of gluconeogenesis (*i.e*., formation of glucose or glycogen from noncarbohydrate sources such as phosphoenolpyruvate).

(*d*) Finally, the oxaloacetate can also condense with acetyl-CoA (Step 1 of the citric acid cycle) and thus initiate the reactions of the citric acid cycle.

Thus, succinate may be *anaplerotic*, giving rise to oxaloacetate, or *gluconeogenic*, giving rise to phosphoenolpyruvate. In this way, net carbohydrate synthesis from fatty acids *via* acetylCoA, which is not accomplished in animals, is achieved in the higher plants and microorganisms. ".....the glyoxylate cycle provides an example par excellence of an anaplerotic sequence in operation" (Mahler and Cordes, 1968).

As may be visualized in Fig. 22-17, the glyoxylate pathway makes use of both mitochondrial and glyoxysomal enzymes.

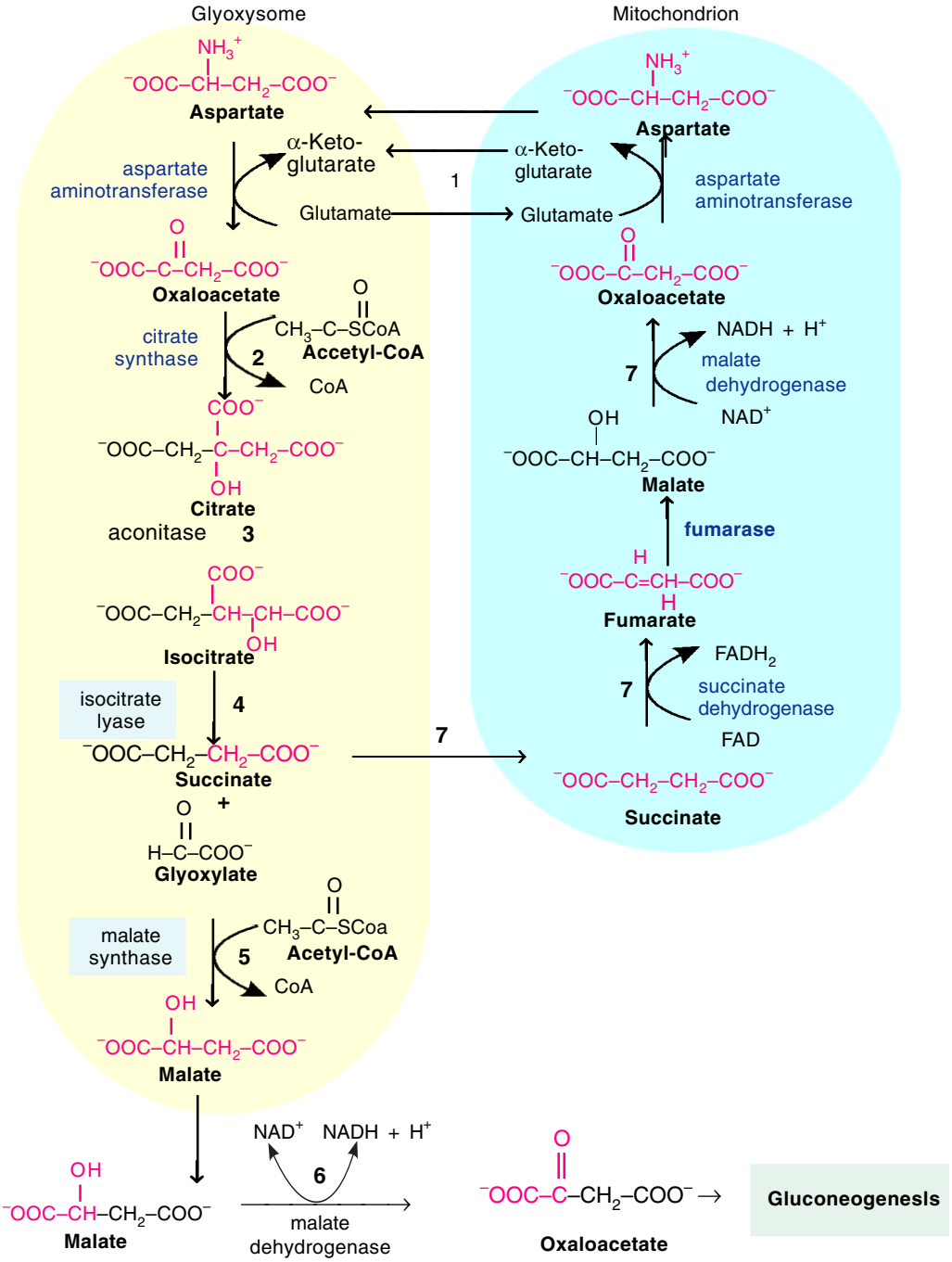


Fig. 22–17. Participation of both mitochondrial and glyoxysomal enzymes in the glyoxylate pathway
Isocitrate lyase and malate synthase, enzymes unique to plant glyoxysomes, are boxed. The pathway results in the net conversion of two acetyl-CoA to oxaloacetate. (1) Mitochondrial oxaloacetate is converted to aspartate, transported to the glyoxysome, and reconverted to oxaloacetate (2) Oxaloacetate is condensed with acetyl-CoA to form citrate. (3) Aconitase catalyzes the conversion of citrate to isocitrate. (4) Isocitrate lyase catalyzes the cleavage of isocitrate to succinate and glyoxylate (5) Malate synthase catalyzes the condensation of glyoxylate with acetyl-CoA to form malate. (6) After transport to the cytosol, malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate, which can then be used in gluconeogenesis (7) Succinate is transported to mitochondrion, where it is reconverted to oxaloacetate *via* the citric acid cycle.

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PROBLEMS

1. What is the $\Delta G^{\circ\prime}$ for the complete oxidation of the acetyl unit of acetyl-CoA by the citric acid cycle ?
2. The citric acid cycle itself, which is composed of enzymatically catalyzed steps, can be thought of essentially as the product of a supramolecular enzyme. Explain.
3. Patients in shock will often suffer from lactic acidosis due to a deficiency of O_2. Why does a lack of O_2 lead to lactic acid accumulation ? One treatment for shock is to administer dichloroacetate, which inhibits the kinase associated with the pyruvate dehydrogenase complex. What is the biochemical rationale for this treatment ?
4. The oxidation of malate by NAD^+ to form oxaloacetate is a highly endergonic reaction under standard conditions ($\Delta G^{\circ\prime}$ = + 7 kcal mol^{-1} (+ 29 kJ mol^{-1})]. The reaction proceeds readily under physiological conditions.
   (*a*) Why ?
   (*b*) Assuming an [NAD^+]/[NADH] ratio of 8 and a pH of 7, what is the lowest [malate]/[oxaloacetate] ratio at which oxaloacetate can be formed from malate ?
5. Propose a reaction mechanism for the condensation of acetyl-CoA and glyoxylate in the glyoxylate cycle of plants and bacteria.
6. The citrate (or any other symmetric compound) cannot be an intermediate in the formation of α-ketoglutarate, because of the asymmetric fate of the label. This view seemed compelling until Alexander Ogston incisively pointed out in 1948 that "it is possible that *an asymmetric enzyme which attacks a symmetrical compound can distinguish between its identical groups."* For simplicity, consider a molecule in which two hydrogen atoms, a group X, and a different group Y are bonded to a tetrahedral carbon atom as a model for citrate. Explain how a symmetric molecule can react with an enzyme in an asymmetric way.
7. Consider the fate of pyruvate labeled with ^{14}C in each of the following positions : carbon 1 (carboxyl), carbon 2 (carbonyl), and carbon 3 (methyl). Predict the fate of each labeled carbon during one turn of the citric acid cycle.

8. Which carbon or carbons of glucose, if metabolized *via* glycolysis and the citric acid cycle, would be most rapidly lost as CO_2 ?

9. Would you expect NAD^+ or CoA-SH to affect the activity of pyruvate dehydrogenase kinase? Briefly explain your answer.

10. Given what you know about the function of the glyoxylate cycle and the regulation of the citric acid cycle, propose control mechansims that might regulate the glyoxylate cycle.

11. Write a balanced equation for the conversion in the glyoxylate cycle of two acetyl units, as acetyl-CoA, to oxaloacetate.

12. FAD is a stronger oxidant than NAD^+ ; FAD has a higher standard reduction potential than NAD^+. Yet in the last reaction of the pyruvate dehydrogenase complex, $FADH_2$ bound to E_3 is oxidized by NAD^+. Explain this apparent paradox.

13. Given the roles of NAD^+/NADH in dehydrogenation reactions and NADPH/$NADP^+$ in reductions, would you expect the intracellular ratio of NAD^+ to NADH to be high or low? What about the ratio of $NADP^+$ to NADPH ? Explain your answers.

14. Oxaloacetate is formed in the last step of the citric acid cycle by the NAD^+-dependent oxidation of L-malate. Can a net synthesis of oxaloacetate take place from acetyl-CoA using only the enzymes and cofactors of the citric acid cycle, without depleting the intermediates of the cycle ? Explain. How is the oxaloacetate lost from the cycle (to biosynthetic reactions) replenished ?

15. α-ketoglutarate plays a central role in the biosynthesis of several amino acids. Write a series of known enzymatic reactions that result in the net synthesis of α-ketoglutarate from pyruvate. Your proposed sequence must not involve the net consumption of other citric acid cycle intermediates. Write the overall reaction for your proposed sequence and identify the source of each reactant.

16. Although oxygen does not participate directly in the citric acid cycle, the cycle operates only when O_2 is present. Why ?

17. Two of the steps in the oxidative decarboxylation of pyruvate (Steps 4 and 5, Fig. 22-) do not involve any of the three carbons of pyruvate yet are essential to the operation of the pyruvate dehydrogenase complex. Explain.

18. Using pyruvate, labeled with ^{14}C in its keto group, *via* the pyruvate dehydrogenase reaction and the TCA cycle, where would the carbon label be at the end of one turn of the TCA cycle ? Where would the carbon label be at the end of the second turn of the cycle ?

19. Explain why, when glucose is the sole carbon source, bacteria grow much more slowly in the absence of O_2 than in the presence of O_2.

20. In which region of a mitochondria are most enzymes of the citric acid cycle located ?

(*a*) outer membrane

(*b*) inner membrane

(*c*) intermembrane space

(*d*) matrix

21. In which of the following organisms would you never find mitochondria ?

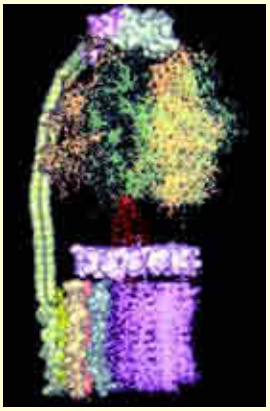
(*a*) a bacterium

(*b*) *Mucor*

(*c*) an yeast

(*d*) *Amoeba*

CONTENTS



A model for mitochondrial F_oF_1-ATP synthetase, a rotating molecular motor

ATP synthesis occurs on F_1 domain, while F_0 domain contains a proton channel. The a, b, α, β, and δ subunits constitute the stator while the c, γ, and ε subunits provide the rotor. Protons flow through the structure causing the rotor to turn, resulting in conformational changes in the β, subunits where ATP is synthesized.

(Courtesy : Drs. Peter L. Pedersen, Young Hee Ko, and Sangjin Hong)

CHAPTER

23

Electron Transport and Oxidative Phosphorylation

INTRODUCTION

All the enzyme-catalyzed steps in the oxidative degradation of carbohydrates, fats and amino acids in aerobic cells converge into electron transport and oxidative phosphorylation, the final stage of cell respiration. This stage consists of flow of electrons from organic substrates to oxygen with the simultaneous release of energy for the generation of ATP molecules. The importance of this final stage of respiration in the human body can be realized by the fact that a normal adult businessman with a 70 kg weight requires about 2,800 kcal of energy per day. This amount of energy can be produced by the hydrolysis of about 2,800/7.3 = 380 mole or 190 kilograms of ATP. However, the total amount of ATP present in his body is about 50 grams. In order to provide chemical energy for the body need, the 50 g of ATP must be broken down into ADP and phosphate and resynthesized thousands of times in a day. *i.e.*, 24 hours.

ELECTRON FLOW AS SOURCE OF ATP ENERGY

Fig. 23–1 shows the electron transport chain in abbreviated form. In each turn of citric acid cycle, 4 pairs

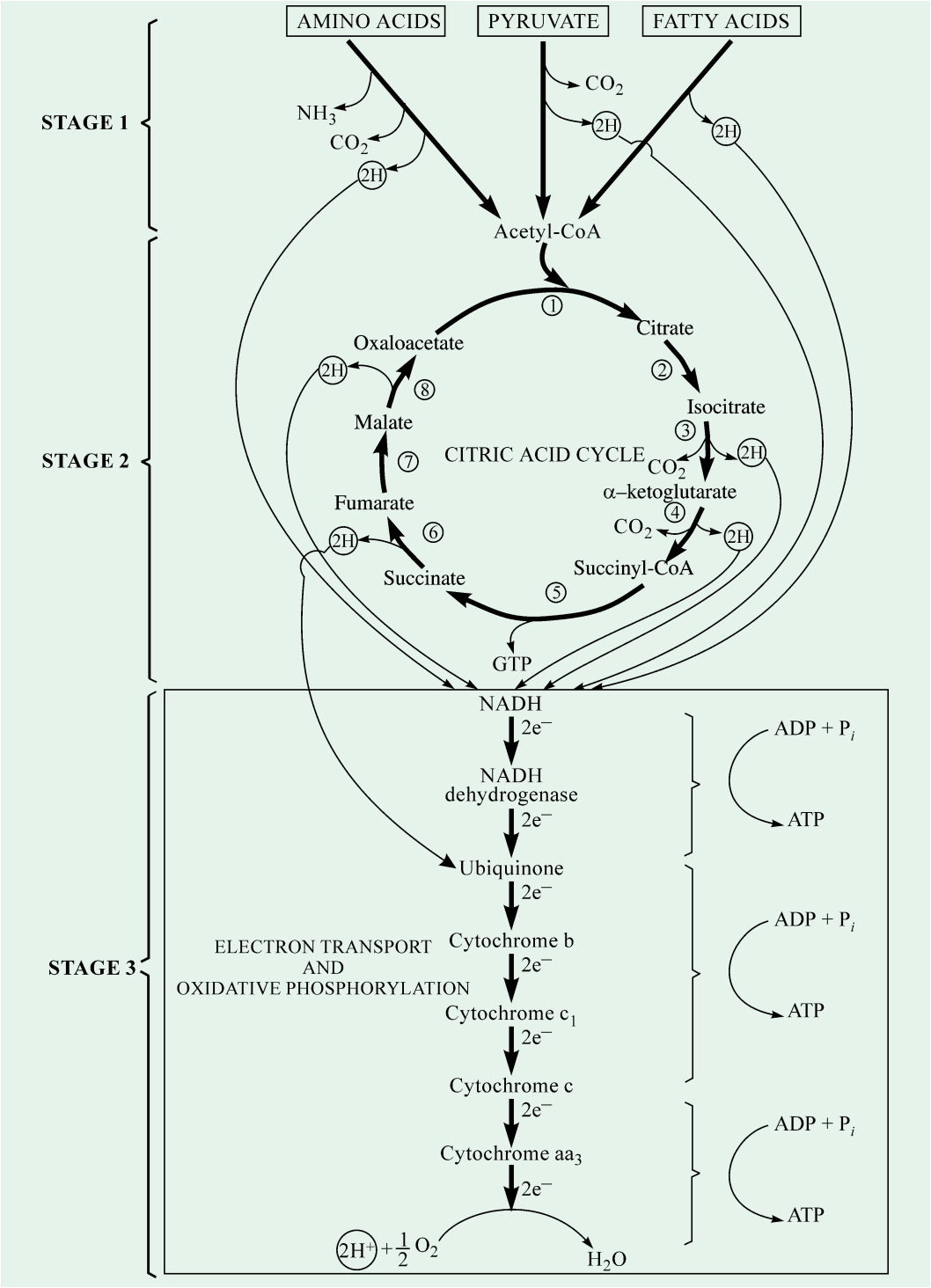


Fig. 23–1. The flow sheet of respiration with special reference to the electron transport and oxidative phosphorylation

The electron transport chain is shown here in abbreviated form. The circled numbers represent the various steps of the citric acid cycle.

of hydrogen atoms are eliminated, one each from isocitrate, a-ketoglutarate, succinate and malate, by the action of specific dehydrogenases. These hydrogen atoms donate their electrons to the electron transport chain and become H^+ ions, which escape into the aqueous medium. These electrons are transported along a chain of electron-carrying molecules to ultimately reach cytochrome aa_3 or cytochrome oxidase, which promotes the transfer of electrons to oxygen, the final electron acceptor in aerobic organisms. As each atom of oxygen accepts two electrons from the chain, two H^+ are taken up from the aqueous medium to form water.

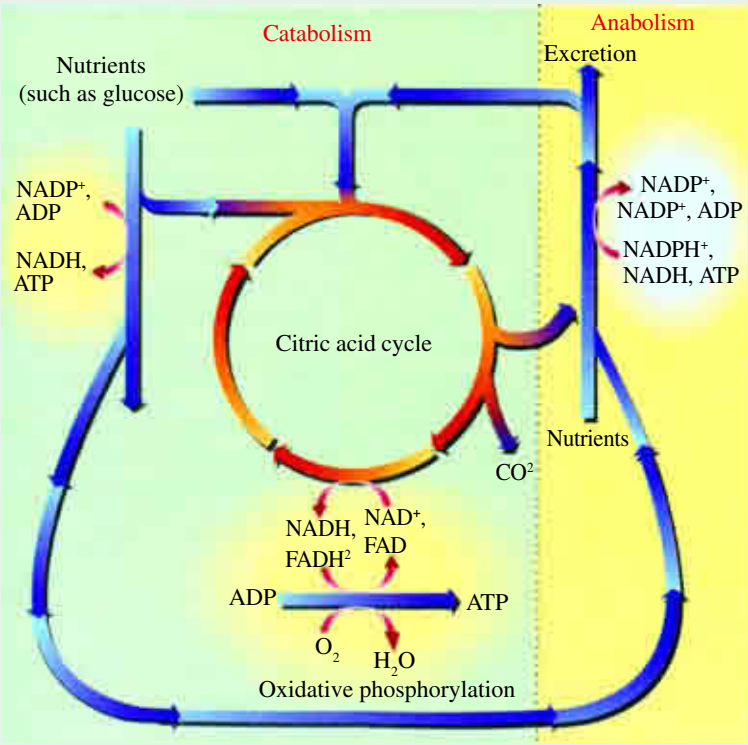


Fig. 23–2. The role of electron transfer and ATP production in metabolism
NAD^+, FAD, and ATP are constantly recycled.

Besides the citric acid cycle, other pairs of hydrogen atoms are also released from the dehydrogenases that act upon pyruvate, fatty acid and amino acids during their degradation to acetyl-CoA and other products. All these hydrogen atoms virtually donate their electrons ultimately to the respiratory chain with oxygen as the terminal electron acceptor.

The respiratory chain consists of a series of proteins with tightly bound prosthetic groups capable of accepting and donating electrons. Each member of the chain can accept electrons from the preceding member and transfer them to the following one, in a specific sequence. The electrons entering the electron-transport chain are energy-rich, but as they pass down the chain step-by-step to oxygen, they lose free energy. Much of this energy is conserved in the form of ATP in the inner mitochondrial membrane. As each pair of electrons passes down the respiratory chain from NADH to oxygen, the synthesis of 3 moles of ATP from ADP and phosphate takes place. The 3 segments of the respiratory chain that provide energy to generate ATP by oxidative phosphorylation are called their **energy-conserving sites**.

The role of electron flow and ATP production is highlighted in Fig- 23-2.

SITE OF OXIDATIVE PHOSPHORYLATION

Mitochondria (Fig. 23–3) are ovate organelles, about 2 μm in length and 0.5 μm in the diameter. Eugene P. Kennedy and Albert L. Lehninger discovered that *mitochondria contain the respiratory assembly, the enzymes of the citric acid cycle and the enzymes of fatty acid oxidation.* Electron microscopic studies by George Palade and Fritjof Sjöstrand have revealed that each mitochondrion (Fig. 23–4) has two membrane systems : an outer membrane and an extensive *inner membrane*, which is highly-folded into a series of internal ridges called *cristae*. Obviously, there are two

compartments in motochondria : the intermembrane space between the outer and inner membranes and the *matrix*, which is bounded by the inner membrane.

The **outer membrane** is freely permeable to most small molecules and ions and contains some enzymes. In contrast, the **inner membrane** is impermeable to nearly all ions and most uncharged molecules and contains the electron-transport chains, succinate dehydrogenase and ATP-synthesizing enzymes. The inner membrane of a single liver mitochondrion may have over 10,000 sets of electron-transport chains and ATP synthetase molecules. The heart mitochondria have profuse cristae and therefore contain about 3 times more sets of electron-transport chains than that of liver mitochondria. The **intermembrane space** contains adenylate kinase and some other enzymes whereas the **matrix** compartment contains most of the citric acid cycle enzymes, the pyruvate dehydrogenase system and the fatty acid oxidation system. It also contains ATP, ADP, AMP, phosphate, NAD, NADP, coenzyme A and various ions such as K^+, Mg^{2+} and Ca^{2+}.

Fig. 23–3. Mitochondria, shown here, are the sites of the citric acid cycle, electron transport, and oxidative phosphorylation.

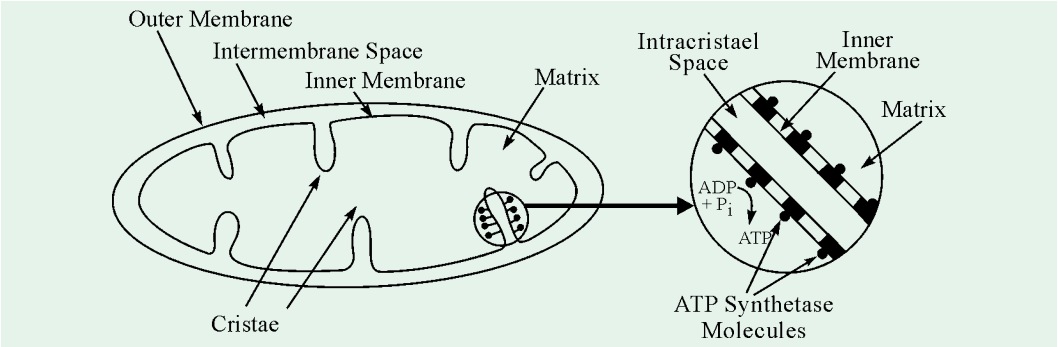


Fig. 23–4. Biochemical anatomy of a mitochondrion

The base piece of ATP synthetase molecules are located with the inner membrane. ATP is made in the matrix, as shown.

ATP SYNTHETASE (=F_oF_1 ATPase)

The mitochondrial inner membrane contains the ATP-synthesizing enzyme complex called *ATP synthetase* or *F_o F_1 ATPase* (Figs. 23–5 and 23-6). This enzyme complex has 2 major components F_0 and F_1 (F for factor). The F_1 component is like a doorknob protruding into the matrix from the inner membrane. It is attached by a stalk to Fo component, which is embedded in the inner membrane and extends across it.

The **subscript of F_o** is not zero but the letter O which denotes that it is the portion of the ATP synthetase which binds the toxic antibiotic, oligomycin. Oligomycin is a potent inhibitor of this enzyme and thus of oxidative phosphorylation.

The F_1 components was first extracted from the mitochondrial inner membrane and purified by Efraim Racker and his collaborators in 1960. During the sonic treatment of the inner mitochondrial membrane (Fig. 23–7), the cristae membranes are fragmented which, later on, reseal to form vesicles called *submitochondrial particles*, in which the F_1 spheres are on the outside, rather than the inside. In other words, the vesicles are "inside out". When these inverted vesicles are treated with urea or trypsin, the F_1 spheres become detached. The isolated F_1 alone cannot make ATP from ADP and inorganic phosphate but it can hydrolyze ATP to ADP and phosphate. It is, therefore, also called as F_1

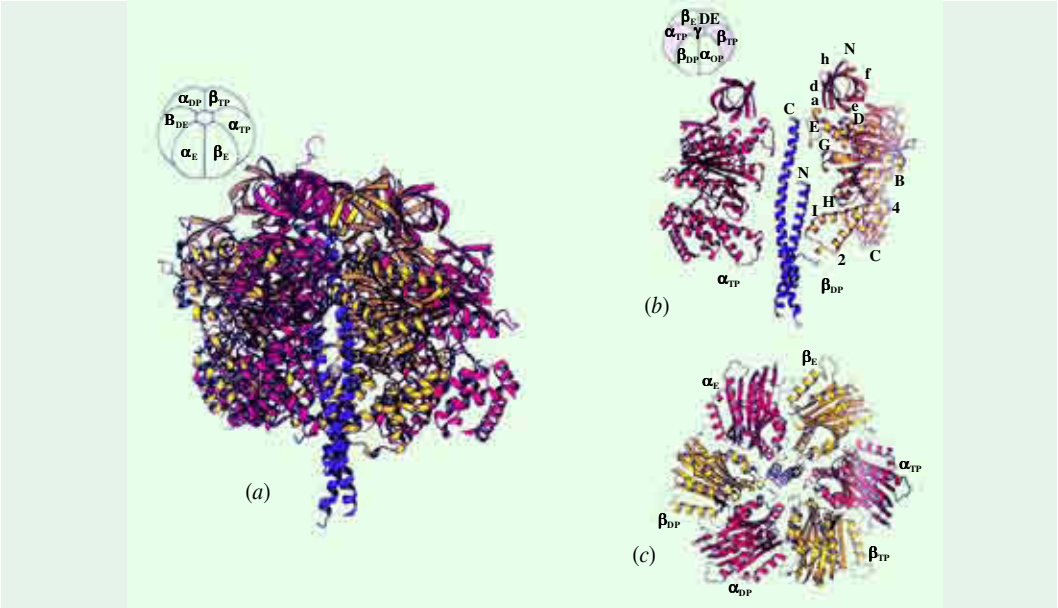


Fig. 23–5. Ribbon model of the 3-dimensional structure of mitochondrial ATP synthase complex (=F_0F_1 ATPase)

(*a*) Side view of F_1 complex structure deduced from the crystal structure. Three α (red) and three β (yellow) subunits alternate around a central shaft, the γ subunit (blue).

(*b*) Side view of F_1 subunit in which two α and β subunits have been removed to reveal the central γ subunit. subunits are colored as indicated for part (*a*).

(*c*) Top view of F_1 complex shows alternating α and β subunits surrounding central γ subunit.

(*Courtesy : Abrahams JP et al, 1994*)

ATPase. The stripped submitochondrial particles (*i.e.*, those lacking F_1 spheres but containing the F_0 component within the inner membrane) can transfer electrons through their electron-transport chain, but they can no longer synthesize ATP. Interestingly enough, Racker found that the addition of these F_1 spheres to the stripped mitochondrial particles restored their capacity to synthesize ATP. Thus, the physiological role of the F_1 component is to catalyze the synthesis of ATP.

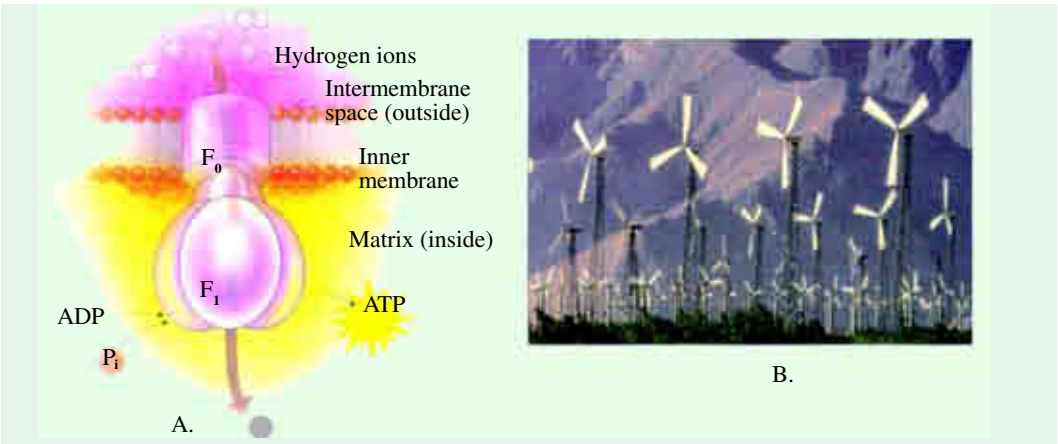


Fig. 23–6. ATP synthase (F_0F_1 complex), the site of ATP synthesis

A. The F_0F_1 complex harnesses energy stored in the proton gradient across the inner mitochondrial membrane. F_o serves as a channel for protons flowing back into the matrix, and F_1 is an enzyme that makes ATP as the protons pass. B. A windmill's blades spin around as it generates electricity. In the same way, the F_1 blades of the F_oF_1 complex spin around as the complex generates ATP.

(*Courtesy : (B) Alan Reininger*)

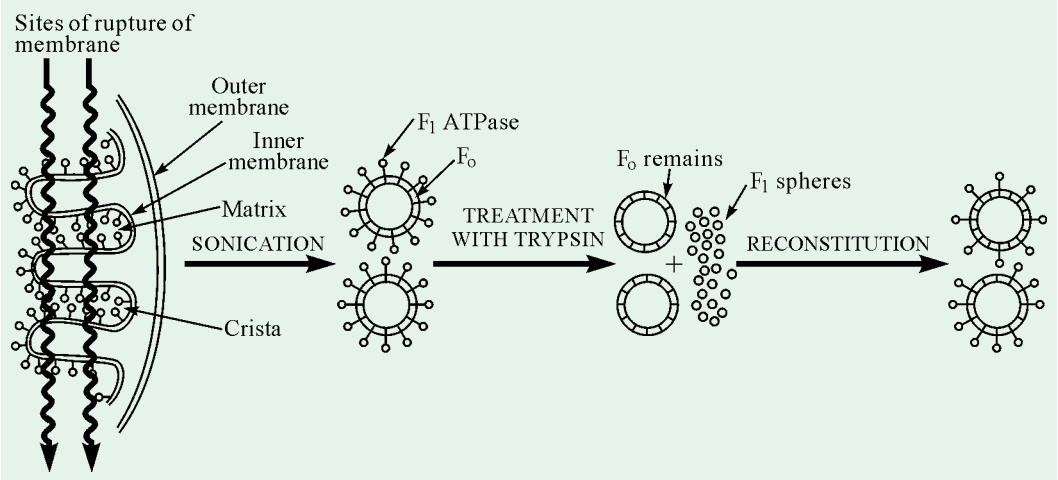


Fig. 23–7. Sonication of the mitochondrial inner membrane

(After Lehninger AL, 1984)

Table 23–1 lists some characteristics of the various components of the mitochondrial ATP-synthesizing complex.

The spheric **F_1 component** (MW = 360 kdal) contains 9 polypeptide chain subunits of five kinds (designated as α, β, γ, δ and ∈) arranged into a cluster. It has many binding sites for ATP and ADP. The cuboidal **F_0 component** is a hydrophobic segment of 4 polypeptide chains. It acts as a base piece and normally extends across the inner membrane. *F_o is the proton channel of the enzyme complex.* The cylindric stalk between F_o and F_1 includes many other proteins. One of them renders the enzyme complex sensitive to oligomycin, an antibiotic that blocks ATP synthesis by interferring with the utilization of the proton gradient. *The stalk is the communicating portion of the enzyme complex.* Fo F1 ATPase is called an ATPase because, in isolated form, it hydrolyzes ATP to ADP plus Pi. However, since its major biological role in intact mitochondria is to produce ATP from ADP and Pi, it is better called ATP synthestase.

Table 23–1. Components of the mitochondrial ATP synthetase

Subunits	*Mass (in kcal)*	*Role*	*Location*
F_1	360	Contains catalytic site for ATP synthesis	Spherical headpiece on matrix side
α	53		
β	50		
γ	33		
δ	17		
∈	7		
F_o	29	Contains proton channel	Transmembrane
	22		
	12		
	8		
F_1 inhibitor	10	Regulates proton flow and ATP synthesis	Stalk between F_0 and F_1
Oligomycin-sensitivity conferring protein (OSCP)	18		
Fc_2 (F_6)	8		

(Adapted from De Pierre and Ernster, 1977)

It is interesting to note that similar phosphorylating units are found inside the plasma membrane of bacteria but outside the membrane of chloroplasts. A noteworthy point is that the proton gradient is from outside to inside in mitochondria and bacteria but in the reverse direction in chloroplasts.

ELECTRON-TRANSFERRING REACTIONS

Chemical reactions in which electrons are transferred from one molecule to another are called **oxidation-reduction reactions** or **oxidoreductions** or **redox reactions**. In fact, the electron-transferring reactions are oxidation-reduction reactions. The electron-donating molecule in such a reaction is called the *reducing agent* (= *reductant*) and the electron-accepting molecule as the *oxidizing agent* (= *oxidant*). The reducing or oxidizing agents function as *conjugate reductant-oxidant pairs* (= redox pairs). The general equation can be written as :

$$\text{Electron donor} \rightleftharpoons e^- + \text{Electron acceptor}$$

A specifie example is the reaction,

$$Fe^{2+} \rightleftharpoons e^- + Fe^{3+}$$

where ferrous ion (Fe^{2+}) is the electron donor and the ferric ion (Fe^{3+}) the electron acceptor. Fe^{2+} and Fe^{3+} together constitute a conjugate redox pair.

Electrons are transferred from one molecule to another in one of the following ways (Lehninger AL, 1984) :

1. *Directly in the form of electrons* — For example, the $Fe^{2+} - Fe^{3+}$ redox pair can transfer an electron to the $Cu^{+} - Cu^{2+}$ pair.

$$Fe^{2+} + Cu^{2+} \longrightarrow Fe^{3+} + Cu^{+}$$

2. *In the form of hydrogen atoms* — A hydrogen atoms consists of a proton (H^+) and a single electron (e^-). The general equation can be written as :

$$AH_2 \rightleftharpoons A + 2e^- + 2H^+$$

in which AH_2 is the hydrogen (or electron) donor, A is the hydrogen acceptor and AH_2 and A together constitute a conjugate redox pair. This redox pair can reduce the electron acceptor B by the transfer of H atoms as follows :

$$AH_2 + B \longrightarrow A + BH_2$$

3. *In the form of a hydride ion*— The hydride ion (: H^-) bears two electrons, as in the case of NAD-linked dehydrogenases."

4. *During direct combination of an organic reductant with oxygen*— In such reactions a product is formed in which the oxygen is covalently incorporated, for example in the oxidation of a hydrocarbon to an alcohol,

$$R{-}CH_3 + \frac{1}{2}O_2 \longrightarrow R{-}CH_2{-}OH$$

where the hydrocarbon is the electron donor and the oxygen atom, the electron acceptor.

All four types of electron transfer occur in cells. The neutral term reducing equivalent is commonly used to designate a single electron equivalent participating in redox reactions, whether it is in the form of an electron *per se*, a hydrogen atom, a hydride ion, or as a reaction with oxygen to yield an oxygenated product. Because biological fuel molecules usually undergo dehydrogenation to lose two reducing equivalents at a time and also because each oxygen atom can accept two reducing equivalents, it is customary to treat the unit of biological oxidations as a pair of reducing equivalents passing from substrate to oxygn.

STANDARD OXIDATION-REDUCTION POTENTIAL

The tendency of a given conjugate redox pair to lose an electron can be specified by a constant variously called as the *standard oxidation-reduction potential, standard redox potential* or simply

standard potential and is denoted by the symbol, E_o'. This is defined as the electromotive force (e.m.f.) in volts given by a responsive electrode placed in solution containing both the electron donor and its conjugate electron acceptor at 0.1 M concentration, 25°C and 7.0 pH. Although standard potentials are given in unts of *volts*, they are often expressed in *millivolts* for convenience. Each conjugate redox pair has a characteristic standard oxidation-reduction potential. By convention, the standard potentials of conjugate redox pairs are expressed as reduction potentials, which assign increasingly negative values to systems having an increasing tendency to lose electrons, and increasingly positive values to systems having an increasing tendency to accept electrons. In other words, the more negative the E_o', the lower is the affinity of the system for electrons and conversely, the more positive the E_o' of a system, the greater is its *electron affinity*. Thus, electrons tend to flow from one redox couple to another in the direction of the more positive system.

Table 23–2 gives the standard redox potentials of some systems useful in biological electron transport. They are listed in order of increasing potential, *i.e.*, in the order of decreasing tendency to lose electrons. Thus, conjugate redox pairs having relatively negative standard potential tend to lose electrons to those lower in the table. For example, when the isocitrate/α-ketoglutarate + CO_2 couple is present in 1.0 concentration, it has a standard potential E_o' of –0.38 V. This redox couple tends to pass electrons to the redox couple NADH/NAD^+, which has a relatively more positive potential in the

Table 23–2. Standard redox potentials, E_o' of some redox pairsparticipating in oxidative metabolism*

Redox couple	E_o' *(in volts)*
Some substrate couples	
Acetyl-CoA + CO_2 + $2H^+$ + $2e^-$ ⟶ Pyruvate + CoA	– 0.48
α-ketoglutarate + CO_2 + $2H^+$ + $2e^-$ ⟶ Isocitrate	– 0.38
3-phosphoglyceroyl phosphate + $2H^+$ + $2e^-$ ⟶ Glyceraldehyde 3-phosphate + Pi	– 0.29
Pyruvate + $2H^+$ + $2e^-$ ⟶ Lactate	– 0.19
Oxaloacetate + $2H^+$ + $2e^-$ ⟶ Malate	– 0.18
Fumarate + $2H^+$ + $2e^-$ ⟶ Succinate	+ 0.03
Components of the electron-transport chain	
$2H^+ + 2e^- \longrightarrow H_2$	**– 0.41**
$NAD^+ + H^+ + 2e^-$ ⟶ NADH	–0.32
$NADP^+ + H^+ + 2e^-$ ⟶ NADPH	– 0.32
NADH dehydrogenase (FMN form) + $2H^+$ + $2e^-$ ⟶ NADH dehydrogenase ($FMNH_2$ form)	– 0.30
Ubiquinone + $2H^+$ + $2e^-$ ⟶ Ubiquinol	+ 0.04
Cytochrome *b* (oxi.) + e^- ⟶ Cytochrome *b* (red.)	+ 0.07
Cytochrome c_1 (oxi.) + e^- ⟶ Cytochrome c_1 (red.)	+ 0.23
Cytochrome *c* (oxi.) + e^- ⟶ Cytochrome *c* (red.)	+ 0.25
Cytochrome *a* (oxi.) + e^- ⟶ Cytochrome *a* (red.)	+ 0.29
Cytochrome a_3 (oxi.) + e^- ⟶ Cytochrome a_3 (red.)	+ 0.55
$1/2O_2 + 2H^+ + 2e^- \longrightarrow H_2O$	**+ 0.82**

* Assuming 1 M concentrations of all compontents, pH = 7.0 and temperature = 25°C.

E_0' refers to the partial reaction written as :

$$\text{Oxidant} + e^- \longrightarrow \text{Reductant}$$

Note the two landmark potentials (in boldface) which are for the $H_2/2H^+$ and the $H_2O/\frac{1}{2}O_2$ couple.

presence of isocitrate dehydrogenase. Conversely, the strongly positive standard potential of H_2O/O_2 couple, 0.82 V, indicates that water molecule has very little tendency to lose electrons to form molecular oxygen. In other words, molecular oxygen has a very high affinity for electrons or hydrogen atoms.

In oxidation systems, the electrons will tend to flow from a relatively electronegative conjugate redox pair, such as NADH/NAD^+ ($E_0' = -0.32$ V), to the more electropositive pair, such as reduced cytochrome *c*/oxidized cytochrome *c* ($E_0' = +0.25$ V). Likewise, they will also tend to flow from the cytochrome *c* redox pair to the water/oxygen pair ($E_0' = +0.82$ V). The greater the difference in the standard potentials between two redox pairs, the greater is the free-energy loss as electrons pass from the electronegative to the electropositive pair. Therefore, when electrons flow down the complete electron-transport chain from NADH to oxygen *via* several electron-carrying molecules, they lose a large amount of free energy.

The amount of free-energy which becomes available as a pair of electrons passes from NADH to O_2 can also be calculated. The standard-free-energy change of an electron-transferring reaction is given by the equation,

$$\Delta G^{\circ\prime} = -nF\,\Delta E_0'$$

where, $\Delta G^{\circ\prime}$ is the standard-free-energy change in calories, *n* is the number of electrons transferred, F is the caloric equivalent of the constant, the faraday (23.062 kcal/V. mol) and $\Delta E_0'$ is the difference between the standard potential of the electron-donor system and that of the electron-acceptor system. The standard-free-energy change as a pair of electron equivalents passes from the NADH/NAD^+ pair ($E_0' = -0.32$ V) to the H_2O/O_2 pair ($E_0' = +0.82$ V) is

$$\begin{aligned}\Delta G^{\circ\prime} &= -2\,(23.062)\,[0.82 - (-0.32)] \\ &= -2 \times 23.062 \times 1.14 \\ &= -52.58 \text{ kcal/mol}\end{aligned}$$

This amount of free energy (*i.e.*, –52.58 kcal) is more than sufficient to bring about the synthesis of 3 moles of ATP, which requires an input of 3 (7.3) = 21.9 kcal under standard conditions.

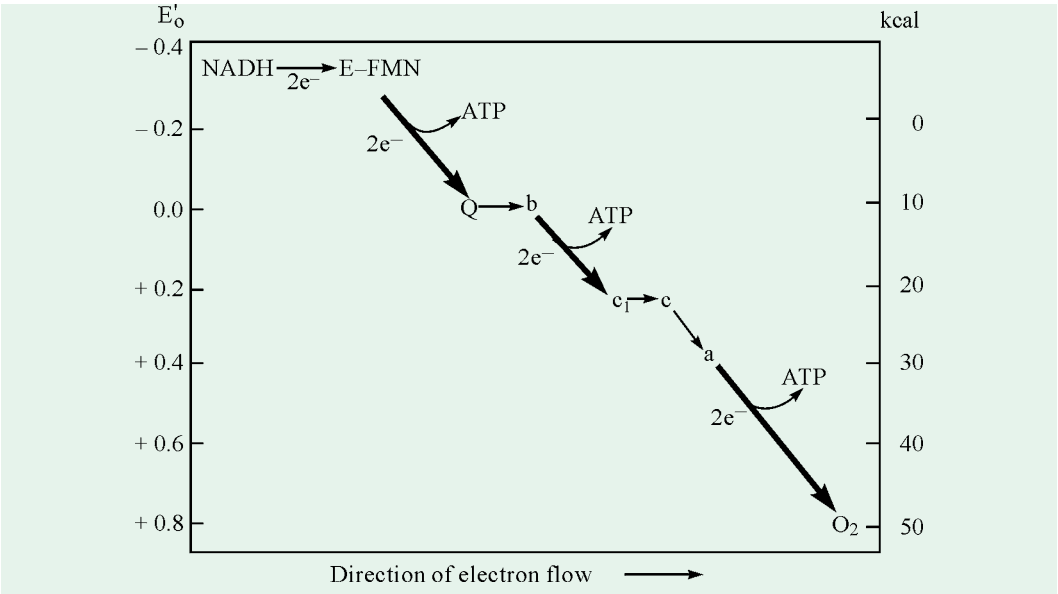


Fig. 23–8. The energy relationships in the respiratory chain of mitochondria

[E-FMN = NADH dehydrogenase; *Q* = ubiquinone; *b*, c_1, *c* and *a* = cytochromes]

Note that there are 3 steps (shown with boldface arrows) in the electron-transport chain in which relatively large decreases in free-energy occur as electrons pass. These are, in fact, the steps that provide free energy for ATP synthesis.

Likewise using the expression $\Delta G^{\circ\prime} = -nF\,\Delta E_0'$, the free-energy changes for individual segments of the electron-transport chains can be calculated from the differences in the standard potentials of the electron-donating redox pair and the electron-accepting pair. Fig. 23–8 is an energy diagram showing (*a*) the standard potentials of the electron carriers of the respiratory chain, (*b*) the direction of electron flow, which is always "downhill" toward oxygen, and (*c*) the relative free-energy change at each step.

ELECTRON CARRIERS

Electrons are transferred from substrates to oxygen through a series of electron carriers— flavins, iron-sulfur complexes, quinones and hemes (Fig. 23–9). The 3 noteworthy features of the electron carriers are enumerated below :

1. Although the figure shows the respiratory chain to have 17 electron carriers, there are some 15 or more chemical groups in the electron-transport chain that can accept or transfer reducing equivalents (hydrogen and electrons) in sequence.
2. All electron carriers, except for the quinones, are prosthetic groups of proteins. They include nicotinamide adenine dinucleotide (NAD), active with various dehydrogenases ; flavin mononucleotide (FMN), in NADH dehydrogenases; ubiquinone or coenzyme Q, which functions in association with one or more proteins ; two different kinds of iron-containing proteins, the iron-sulfur centres (Fe-S) and the cytochromes ; and copper of cytochrome aa_3.
3. Almost all the electron-carrying proteins are water insoluble and are embeded in the inner mitochondrial membrane.

1. Pyridine Nucleotides

Most of the electron pairs entering the respiratory chains arise from the action of dehydrogenases that utilize the coenzymes NAD^+ or $NADP^+$ (Fig. 23–6) as electron acceptors. As a group, they are designated as NAD(P)-linked dehydrogenases. They catalyze the reversible reactions of the following general types :

Reduced substrate + NAD^+ $\rightleftharpoons$ Oxidized substrate + NADH + H^+

Reduced substrate + $NADP^+$ $\rightleftharpoons$ Oxidized substrate + NADPH + H^+

Most of the pyridine-linked dehydrogenases are specific for NAD^+ (refer Table 23–3). However, certain others require $NADP^+$ as electron acceptor, such as glucose 6-phosphate dehydrogenase. Very few, such as glutamate dehydrogenase can react with either NAD^+ or $NADP^+$. Some pyridine-linked dehydrogenases are located in the mitochondria, some in the cytosol and still others in both.

The pyridine-linked dehydrogenases remove 2 hydrogen atoms from their substrates. One of these is transferred as a hydride ion (: H^-) to the NAD^+ or $NADP^+$; the other appears as H^+ in the medium. Each hydride ion carries two reducing

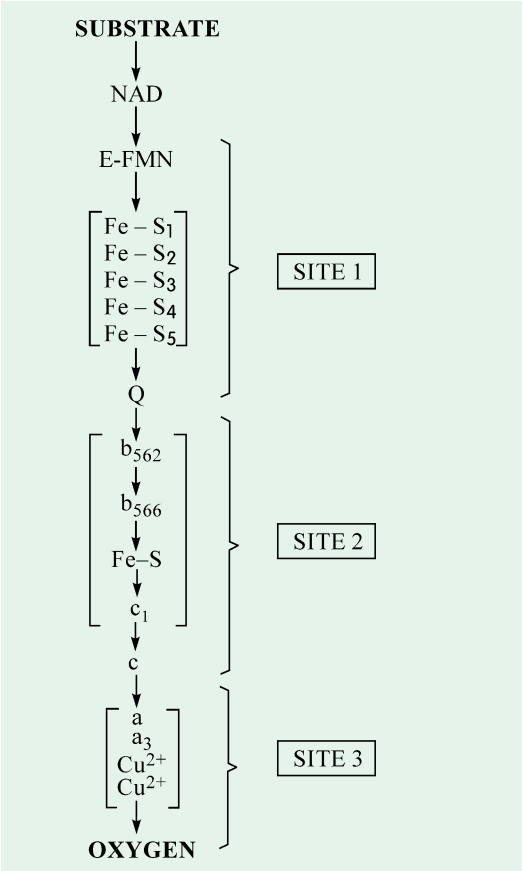


Fig. 23–9. The complete set of electron carriers of the respiratory chain

The precise sequence and function of all the oxidation-reduction centres is not exactly known.

equivalents : one is transferred as a hydrogen atom to C_4 of the nicotinamide ring, the other as an electron to the ring nitrogen (Fig. 23–10).

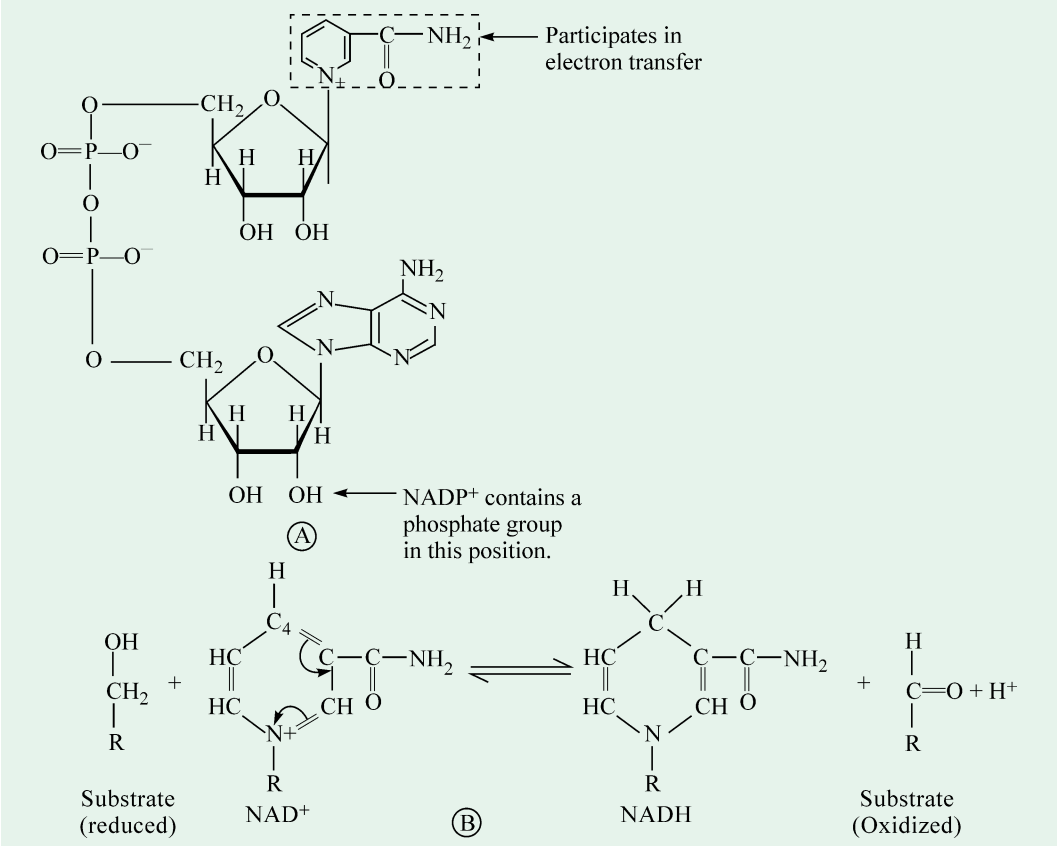


Fig. 23–10. Nicotinamide adenine dinucleotide (NDA^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$)

Ⓐ Oxidized forms of NAD^+ and $NADP^+$; Ⓑ Reduction of the nicotinamide ring of NAD^+ by substrate.

Table 23–3. Some important reactions catalyzed by NAD (or NADP)-linked dehydrogenases

Reaction	*Location*
NAD-linked :	
Isocitrate + NAD+ $\rightleftharpoons$ α-ketoglutarate + CO_2 + NADH + H^+	Mitochondria
α-ketoglutarate + CoA + NAD^+ $\rightleftharpoons$ Succinyl-CoA + CO_2 + NADH + H^+	Mitochondria
L-malate + NAD^+ $\rightleftharpoons$ Oxaloacetate + NADH + H^+	Mitochondria and cytosol
Pyruvate + CoA + NAD^+ $\rightleftharpoons$ Acetyl-CoA + CO_2 + NADH + H^+	Mitochondria
Glyceraldehyde 3-phosphate + Pi + NAD $\rightleftharpoons$ 1, 3-diphosphoglycerate + NADH + H^+	Cytosol
Lactate + NAD^+ $\rightleftharpoons$ Pyruvate + NADH + H^+	Cytosol
NADP-linked :	
Isocitrate + $NADP^+$ $\rightleftharpoons$ α-ketoglutarate + CO_2 + NADPH + H^+	Mitochondria and cytosol
Glucose 6-phosphate + $NADP^+$ $\rightleftharpoons$ 6-phosphogluconate + NADPH + H^+	Cytosol
NAD or NADP:	
L-glutamate + H_2O + NAD^+ ($NADP^+$) $\rightleftharpoons$ α-ketoglutarate + NH_3 + NADH(NADPH) + H^+	Mitochondria

Since most dehydrogenases in cells transfer H atoms from their substrates to NAD^+, this coenzyme *collects* pairs of reducing equivalents from many different substrates, in one molecular form, NADH (Fig. 23–11). Ultimatley, NAD^+ can also collect reducing equivalent from substrates acted upon by NADP-linked dehydrogenases in the presence of *pyridine nucleotide transhydrogenase.*

$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH$$

2. NADH Dehydrogenase (=NADH-Q Reductase)

The transfer of electrons from NADH in the mitochondrial matrix to ubiquinone in the membrane core, and the accompanying pumping of protons, is catalyzed by a highly organized enzyme complex, *NADH dehydrogenase*. This complex includes a flavoprotein and iron-sulfide proteins as electron carriers. In the next step of electron transfer, a pair of reducing equivalents is transferred from NADH to NADH dehydrogenase. In this reaction, the tightly bound prosthetic group of NADH dehydrogenase becomes reduced. This prosthetic group is flavin mononucleotide (FMN), which contains a molecule of vitamin B_2 or riboflavin.

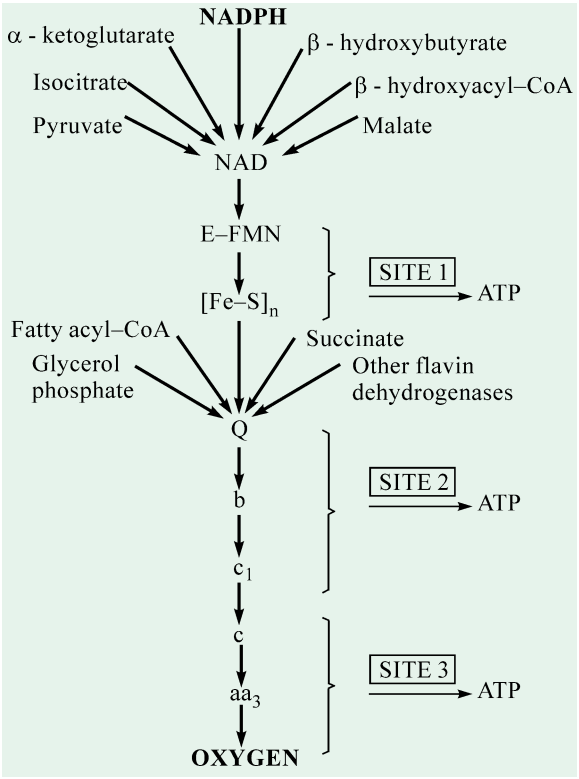
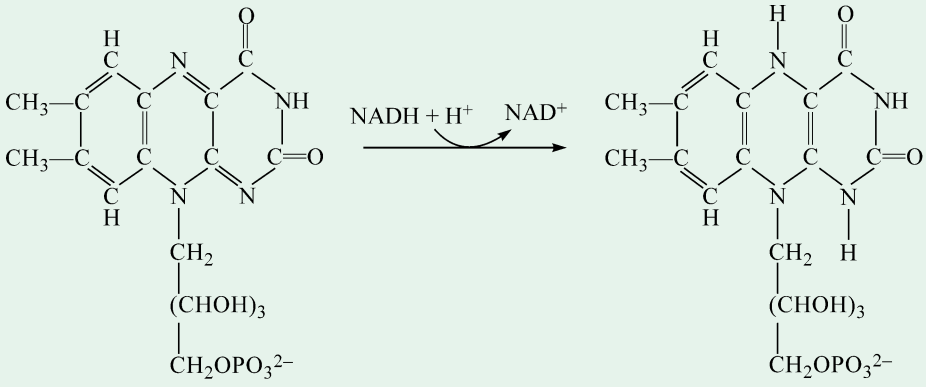


Fig. 23–11. The collecting function of nicotinamide adenine dinucleotide (NAD) and ubiquinone (Q)

Pairs of reducing equivalents collected from flavin dehydrogenases do not pass through the first phosphorylation site and thus give rise to only two ATP molecules.



NADH dehydrogenase is a complex and highly organized flavoprotein enzyme and consists of at least 16 polypeptide chains. It is located in the inner mitochondrial membrane. Transfer of two reducing equivalents from NADH to NADH dehydrogenase (here designated as E-FMN) reduces the FMN to $FMNH_2$ as follows :

$$NAD + H^+ + E—FMN \rightleftharpoons NAD^+ + E—FMNH_2$$

The electrons are then transferred from $FMNH_2$ to a series of iron-sulfur complexes (abbreviated as Fe—S), the second type of prosthetic group in NADH dehydrogenase. The iron is not a part of a heme group and so iron-sulfur proteins were referred to in the older literature as nonheme iron proteins (NHI proteins). Recall that Fe—S centres are also associated with succinate dehydrogenase (see page 400). Three principal types of Fe—S complexes are known (Fig. 23-12). In all of these, the iron atoms are chelated with sulfur atoms, which are in part supplied by cysteine residues in the associated protein and in part as inorganic sulfide ions. The number of iron and acid-labile-sulfur atoms in these complexes is always equal.

1. $FeCys_4$ (or FeS) type—In this simplest kind, a single iron atom is tetrahedrally coordinated to the sulfhydryl groups of 4 cysteine residues of the protein ; there being no inorganic sulfides.

2. $Fe_2S_2Cys_4$ (or Fe_2S_2) type— This contains 2 iron atoms and 2 inorganic sulfides, in addition to 4 cysteine residues. The bonding occurs in such a manner that a total of 4 sulfur atoms is linked to each iron atom.

3. $Fe_4S_4Cys_4$ (or Fe_4S_4) type — This contains 4 iron atoms, 4 inorganic sulfides and 4 cysteine residues. Here also, 4 sulfur atoms are linked to each iron atom.

The iron atoms in these complexes can be in the reduced (Fe^{2+}) or oxidized (Fe^{3+}) state. An important feature of the iron-sulfur proteins is that their relative affinity for electrons can be varied over a wide range by changing the nature of the polypeptide chain. Some are relatively strong oxidizing agents ; others are powerful reducing agents — even stronger than NADH. NADH dehydrogenase contains both the $Fe_2S_2Cys_4$ and $Fe_4S_4Cys_4$ types of complexes.

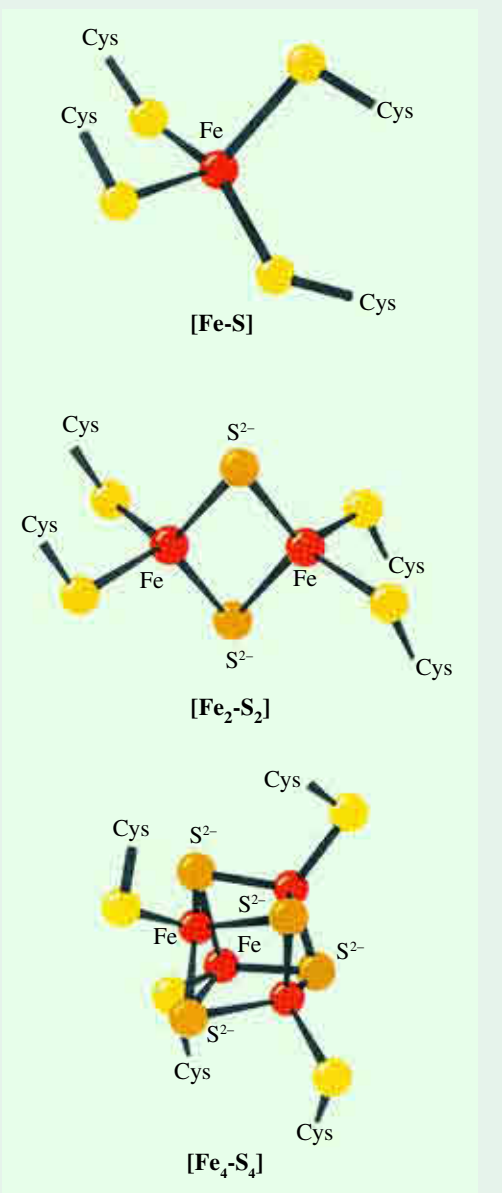


Fig. 23–12. Three principal forms of iron-sulfide proteins.

3. Ubiquinone (=Coenzyme Q)

The next carrier of reducing equivalents in the respiratory chain is ubiquinone (UQ), a name reflecting its ubiquitous nature, as it occurs virtually in all cells. It was formerly called as **coenzyme Q** (Q for quinone) and abbreviated as CoQ or simply Q.

Ubiquinone is actually a group of compounds, all containing the same quinone structure but substituted with a long side chain composed of varying numbers (from 6 to 10) of isoprene units (=prenyl groups), linked head to tail. For example, certain microorganisms contain 6 isoprene units, and in which case the compound is referred to as Q_6 or CoQ_6 or also as UQ_{30}, where the subscript number 30 represents the total number of carbon atoms in the side chain. However, the most common form in mammals contains 10 isoprene units, and when its designation is Q_{10} or CoQ_{10} or UQ_{50}. The

isoprenoid tail makes ubiquinone highly nonpolar which enables it to diffuse readily in the hydrocarbon phase of the inner mitochondrial membrane. *Ubiquinone is the only electron carrier in the respiratory chain that is not tightly bound or covalently attached to a protein.* In fact, it serves as a highly mobile carrier of electrons between the flavoproteins and the cytochromes of the electron-transport chain.

The closely related plastoquinones, which function as an analogous carrier of electrons in photosynthesis, differ from ubiquinones in the alkyl substituents of the benzene ring : two $-CH_3$ groups instead of two $-OCH_3$ and H instead of $-CH_3$. Plastoquinones B and C carry one hydroxyl group in the side chain.

Like other quinones, the ubiquinones may be reduced one electron at a time through the semiquinone free radical or may be reduced directly to the quinol by two electrons (Fig. 23–13).

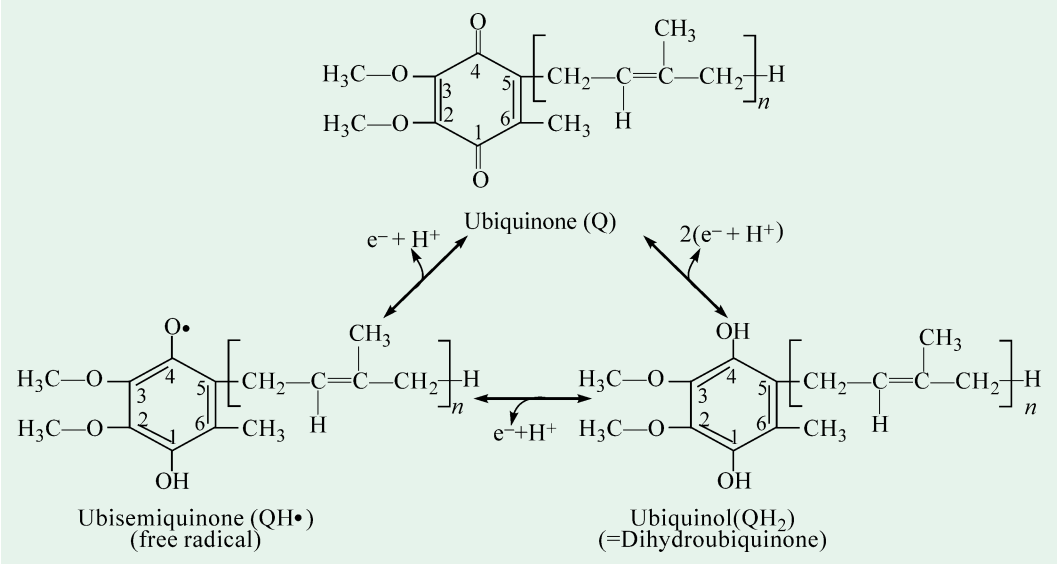


Fig. 23–13. Reduction of ubiquinone

When reduced NADH dehydrogenase (E–$FMNH_2$) donates its reducing equivalents via the Fe—S centres to ubiquinone, the latter becomes reduced to ubiquinol (QH_2) and the oxidized form of NADH dehydrogenase is regenerated.

$$E—FMNH_2 + Q \rightleftharpoons E–FMN + QH_2$$

The function of ubiqunione is to collect reducing equivalents not only from NADH dehydrogenase but also from other flavin-linked dehydrogenases of mitochondria (see Fig. 23–10).

4. Cytochromes

The enzyme complex catalyzing the oxidation of ubiquinol (QH_2) contains an iron-sulfide protein of Fe_2S_2 Cys_4 type and 2 types of cytochromes. The cytochromes are electron-transferring, red or brown proteins that contain a heme prosthetic group and act in sequence to carry electrons from ubiquinone to molecular oxygen. They are, thus, *heme proteins* (or *hemoproteins*) like hemoglobin but, unlike hemoglobin, their iron atoms are oxidized and reduced to transfer electrons between other compounds. The cytochromes were discovered in 1886 by MacMunn, a Scottish physician who also named them as *histohematin* and *myohematin*, since they appeared to him to be related to heme and hemin. But their role in biologic oxidation was first shown by David Keilin in 1925. He also renamed these pigments as 'cytochromes'. The iron atom in cytochromes alternates between a reduced ferrous (Fe^{2+}) state and an oxidized ferric (Fe^{3+}) state during electron transport. *A heme group, like an Fe – S centre, is one-electron carrier, in contrast with NADH, flavins and ubiquinone, which are two-electron carriers.*

There are 5 types of cytochromes between ubiquinol (QH_2) and oxygen in the electron-transport chain. Each type is given a letter designation— *a, b, c* and so on, based on the differences in their light-absorption spectra — the form absorbing at the longest wavelength called cytochrome *a*, that absorbing the next longest wavelength called cytochrome *b*, and so on. *Unfortunately, the order of wavelength does not correspond to the physiological sequence in which they function :*

$$b \rightarrow c_1 \rightarrow c \rightarrow a \rightarrow a_3$$

The subscript numbers were added as new individual cytochromes within the same type were found, *i.e.*, those with similar prosthetic groups but with different apoproteins.

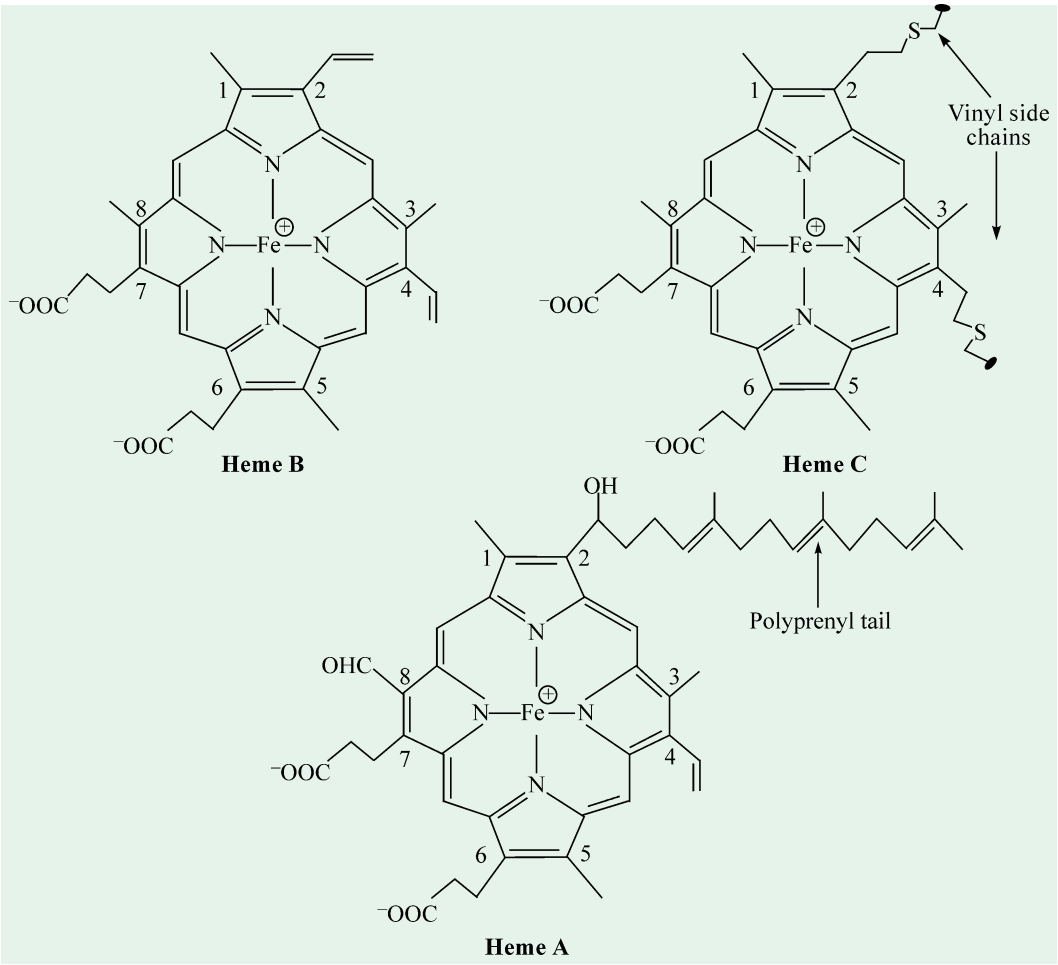


Fig. 23–14. The three principal types of cytochrome hemes

Heme B is the prosthetic group of cytochrome *b*; heme C, that of cytochromes *c* and c_1; and heme A, that of cytochromes *a* and a_3.

The various cytochromes differ from each other in the nature of the prosthetic group and its mode of attachment to the apoprotein part (Fig. 23–14). The prosthetic group of cytochromes *b*, *c* and c_1 is iron-protoporphyrin IX, commonly called *heme* or *hemin*. Heme is also the prosthetic group of myoglobin, hemoglobin, catalase and peroxidase. In cytochrome *b*, the heme is not covalently bonded to the protein, whereas in cytochromes *c* and c_1, the heme is covalently attached to the protein by thioether linkages. These linkages are formed by the addition of the sulfhydryl groups of two cysteine residues to the vinyl groups of the heme. The type of heme present in cytochrome *b* is called heme *B* and the one present in cytochromes *c* and c_1 as heme *C*.

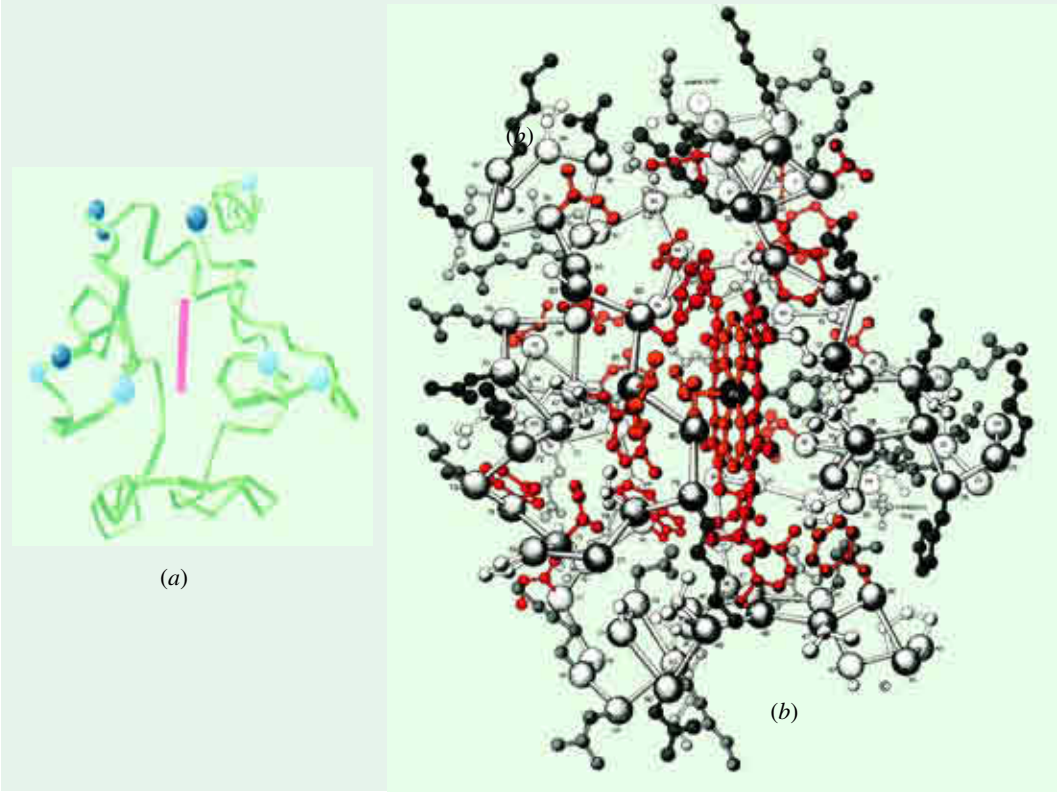


Fig. 23–15. Three dimensional structure of the protein, cytochrome *c*

(*a*). **Ribbon model.** The diagram shows the Lys residues involved in intermolecular complex formation with cytochrome *c* oxidase or reductase as inferred from chemical modification studies. Dark and light blue balls, respectively, mark the positions of Lys residues whose ε-amino groups are strongly and less strongly protected by cytochrome *c* oxidase and reductase against acetylation by acetic anhydride. Note that these Lys residues form a ring around the heme (*solid bar*) on one face of the protein.

(*b*). **Ball-and-stick model.** Amino acids with nonpolar, hydrophobic side chains (color) are found in the interior of the molecule, where they interact with one another. Polar, hydrophilic amino acid side chains (grey) are on the exterior of the molecule, where they interact with the polar acqueous solvent.

(*Courtesy : (a) MS Mathews, and (b) Irving Geis*)

The cytochromes *a* and a_3 have a different iron-porphyrin prosthetic group, called heme *A*. It differs from the heme in cytochromes *c* and c_1 in that a formyl group replaces one of the methyl groups, and a hydrophobic polyprenyl chain replaces one of the vinyl groups. Cytochromes *a* and a_3 are the terminal members of the respiratory chain. They exist as complex, which is sometimes called cytochrome oxidase. The cytochrome aa_3 complex, thus, differs from other cytochromes as it contains 2 moles of highly-bound heme *A*. Moreover, cytochrome aa_3 also contains 2 essential copper atoms. It is the terminal member of the electron-transport chain and was first identified by Warburg as *Atmungsferment*.

Cytochrome *c* is the best known of cytochromes. It is the only electron-transport protein that can be separated from the inner mitochondrial membrane by gentle treatment. The solubility of this peripheral membrane protein in water has facilitated its purification and crystallization. It is a small protein (MW = 12,500) with an iron-porphyrin group (or *heme C*) covalently attached to its single polypeptide chain, containing about 100 amino acid residues (104 in a fish called tuna) in most species. Cytochrome *c* from tuna (Fig. 23–15) is roughly spherical, with a diameter of 34 Å. The heme group is surrounded by many closely-packed hydrophobic side chains. The iron atom is bonded to the sulfur atom of a methionine residue and to the nitrogen atom of a histidine residue. The

hydrophobic nature of the heme environment makes the redox potential of cytochrome *c* more positive, corresponding to a higher electron affinity.

The overall structure of the cytochrome *c* molecule resembles that of a shell, one residue thick surrounding the heme. The side chains make up the interior of the shell. The main polypeptide chain comes next, followed by charged side chains on the surface. *There is a very little a-helix and no* β-*pleated sheet*. In essence, the polypeptide chain is coiled around the heme. Residues 1 to 47 are on the histidine-18 side of the heme (called the right side), and residues 48 to 91 are on the methionine-80 side (called the left side). The remaining 92 to 104 residues come back across the heme to the right side. Cytochorme *c* is an ancient protein, since its amino acid sequence has many points of similarity in all eukaryotes— microbes, plants and animals.

ELECTRON-TRANSPORT COMPLEXES (= Complexes of the Respiratory Chain)

It is now a well-established fact that the electron carriers in the respiratory chain function in a specific sequence. The following evidences support the statement :

1. Firstly, as expected their standard redox potentials (refer Table 23–2 and Fig. 23–6) are more positive going toward oxygen, since electrons tend to flow from electronegative to electropositive systems, causing a decrease in free energy.
2. Secondly, each member of the chain is specific for a given electron donor and acceptor. For instance, NADH can transfer electrons to NADH dehydrogenase but cannot transfer them directly to cytochrome b or to cytochrome *c*.
3. Lastly, four structured complexes of functionally related electron carriers have been isolated from mitochondrial membrane (refer Fig. 23–16) :

Complex I consists of NADH dehydrogenase and its iron-sulfur centres, which are closely linked in their function. Complex I carries out the following characteristic reaction :

$$NADH + H^+ + CoQ \xrightarrow[\text{Complex I}]{\text{NADH : CoQ (oxido) reductase}} NAD^+ + CoQH_2$$

Complex II consists of succinate dehydrogenase and its iron-sulfur centres. This complex carries out the following characteristic reaction :

$$\text{Succinate} + CoQ \xrightarrow[\text{Complex II}]{\text{Succinate : CoQ (oxido) reductase}} \text{Fumarate} + CoQH_2$$

Complex III consists of cytochromes *b* and *c*, and a specific iron-sulfur centre. This brings about the following characteristic reaction :

$$CoQ\,H_2 + 2 \text{ cyt } c\ (Fe^{3+}) \xrightarrow[\text{Complex III}]{\text{Hydro-CoQ : Cytochrome (oxido) reductase}} CoQ + 2 \text{ cyt } c\ (Fe^{2+})$$

Complex IV consists only of cytochromes *a* and a_3, which is sometimes called as cytochrome oxidase. This brings about the following characteristic reaction :

$$4 \text{ cyt } c\ (Fe^{2+}) + O_2 \xrightarrow[\text{Complex IV}]{\text{Cytochrome oxidase (Cytochrome c : O}_2\text{oxido reductase)}} 4 \text{ cyt } c\ (Fe^{3+}) + H_2O$$

Ubiquinone is the connecting link between complexes I , II and III, and cytochrome *c* connects the complexes. III and IV. It is evident that in the presence of 2 mobile carriers (CoQ and cytochrome *c*), this accounts for all the oxidoreductions of the mitochondrial electron transport system. Thus, complex I plus complex III reconstitute the mitochondrial NADH : cytochrome *c* (oxido) reductase ; complex II plus III, the mitochondrial succinate : cytochrome *c* (oxido) reductase ; complex I plus III

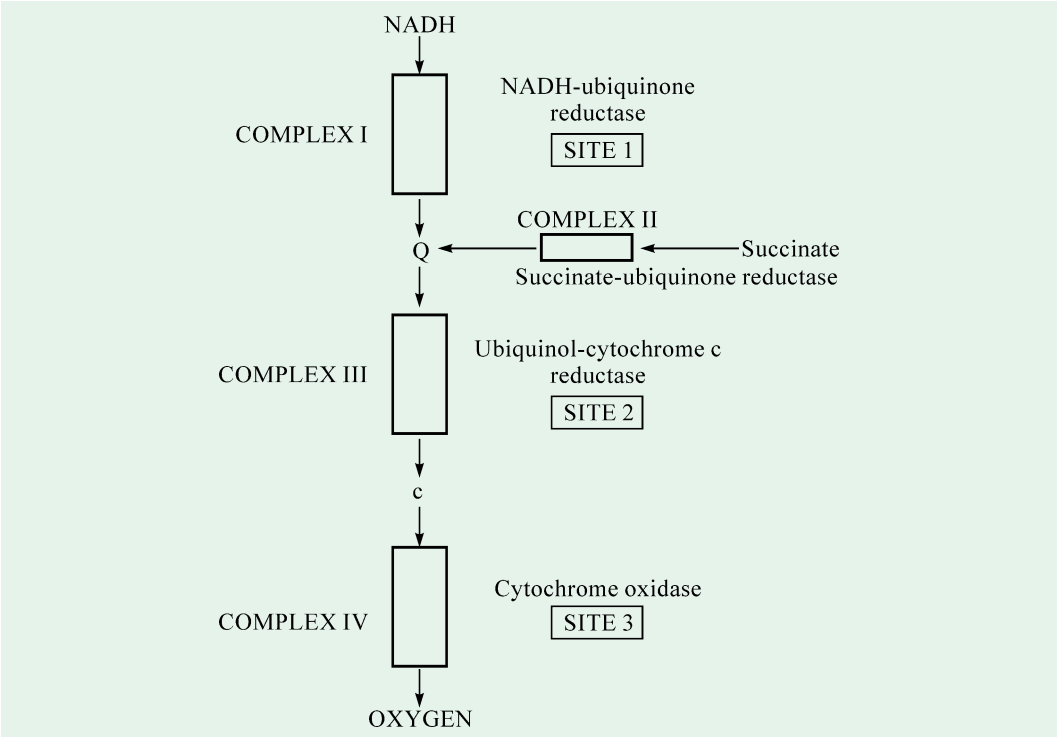


Fig. 23–16. The electron transport complex

The various complexes can be isolated as functional assemblies.

plus IV, the mitochondrial succinoxidase ; and finally complex I plus II plus III plus IV, the complete electron-transport sequence, *i.e.*, a combined NADH and succinoxidase equation :

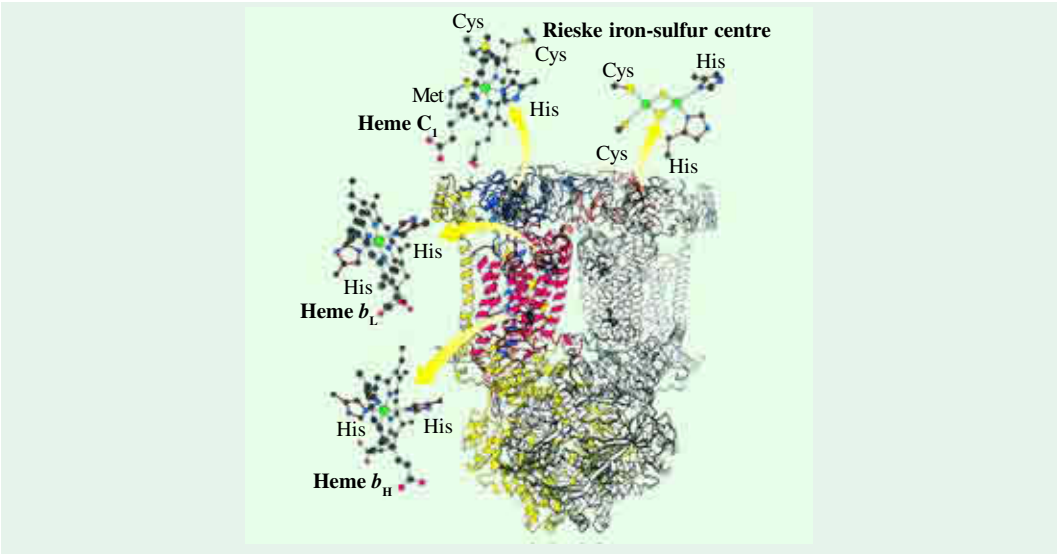
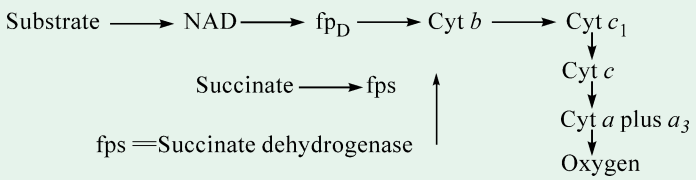
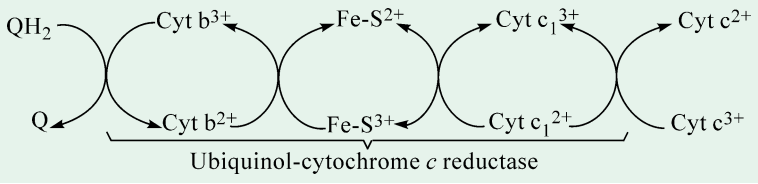


Fig 23–17. Structure of Q-cytochrome c oxidoreductase or cytochrome bc_1 complex or complex III

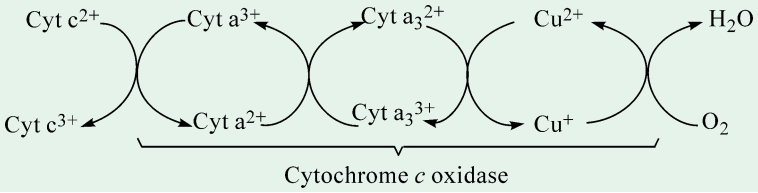
This enzyme is a dimer of identical monomers (*i.e.*, homodimer), each with 11 distinct polypeptide chains. The major prosthetic groups, three hemes and a 2Fe-2S cluster, mediate the electron-transfer reactions between quinones in the membrane and cytochrome c in the intermembrane space. The enzyme protrudes 75 Å into the matrix and 38 Å into the intermembrane space.



The ubiquinol-cytochrome *c* reductase complex (Fig 23-17) transfers electrons from ubiquinol (QH_2) to cytochrome *c*.



Reduced cytochrome *c* then transfers its electrons to the cytochrome *c* oxidase complex (Fig. 23-18). The role of cytochrome *c* is analogous to that of coenzyme Q, *i.e.*, it is a mobile carrier of electrons between different complexes in the respiratory chain.



Electrons are transferred to the cytochrome *a* moiety of the complex, and then to cytochrome a_3, which contains copper. This copper atom alternates between an oxidized (2+) form and a reduced (1+) form as it transfers electrons from cytochrome a_3 to molecular oxygen. *The formation of water is a four-electron process,* whereas heme groups are one-electron carriers. It is not yet certain as to how four electrons converge to reduce a molecule of oxygen.

$$O_2 + 4H^+ + 4e^- \longrightarrow 2\,H_2O$$

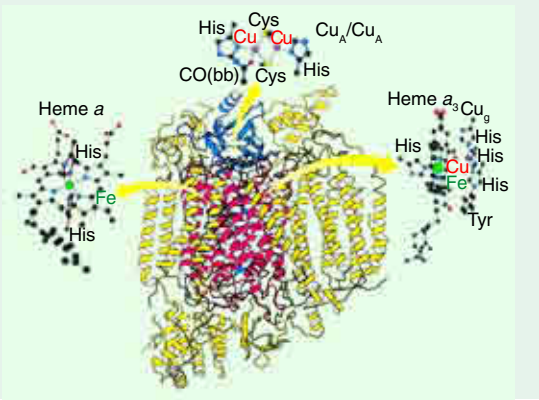


Fig. 23–18. Three dimensional structure of cytochrome *c* oxidase (complex IV)

This enzyme consists of a dimer in which each monomer is composed of 13 different polypeptide chains. The major prosthetic groups include Cu_A/Cu_A, heme *a*, and heme a_3-CuB. Heme a_3-Cu_B is the site of the reduction of oxygen to water. CO(bb) is a carbonyl group of the peptide backbone.

After the cytochrome *a* component receives electrons from cytochrome *c* and thus becomes reduced to the ferrous (Fe^{2+}) form, it passes its electrons to cytochrome a_3. Reduced cytochrome a_3 then passes electrons to molecular oxygen. Participating with the two heme groups in this process are 2 bound copper atoms, which undergo cuprous—cupric redox changes ($Cu^+ - Cu^{2+}$) in their function. This is an important and complex step in electron transport, since 4 electrons must be passed almost simultaneously to O_2 to yield two H_2O molecules, with uptake of 4 H^+ from the aqueous medium. *Of all the members of the electron-transport chain, only cytochrome aa_3 can react directly with oxygen.* The complete electron transport chain along with the respiratory complexes is presented in Fig. 23-19.

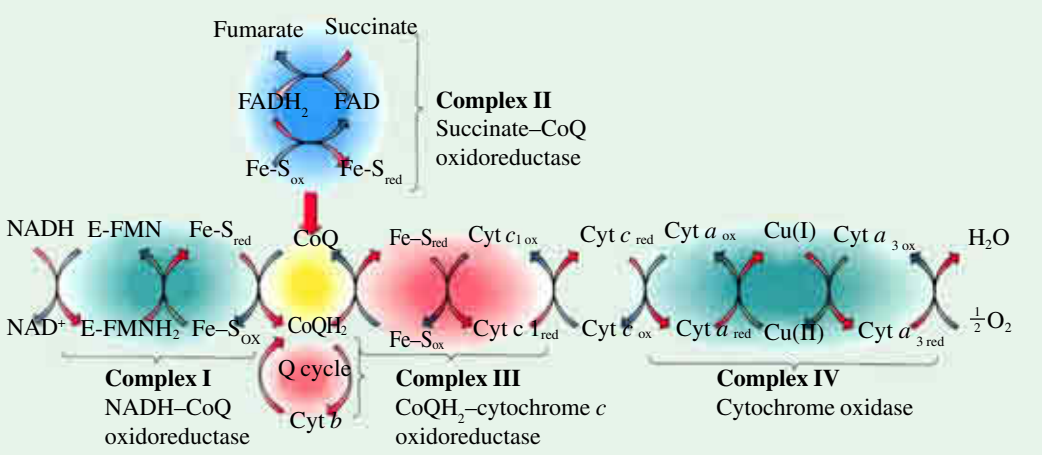


Fig. 23–19. The electron transport chain, showing the respiratory complexes
In the reduced cytochromes, the iron is in the Fe(II) oxidation state, while in the oxidized cytochromes, the oxygen is in the Fe(III) oxidation state

INCOMPLETE REDUCTION OF OXYGEN (=Toxic Metabolites of Oxygen)

It is quite important for the cell that the O_2 molecule be completely reduced to 2 molecules of H_2O by accepting 4 electrons. If, however, O_2 is only partially reduced by accepting 2 electrons, *hydrogen peroxide* (H_2O_2) is formed and if O_2 accepts only one electron, the product formed is the *superoxide radical* ($:O_2^-$). Hydrogen peroxide and superoxide are extremely toxic to cells as they attack the unsaturated fatty acid components of membrane lipids, thus damaging severely the membrane structure. Superoxide is especially dangerous. It does not itself react readily with most cellular constituents, but it will spontaneously combine with peroxides to form *hydroxyl radicals* (OH) and *singlet oxygen* ($^1O_2$), which are disruptively reactive :

$$:O_2^- + H_2O_2 \longrightarrow HO + {}^1O_2 + OH^-$$

The reduction of oxygen, which is also called as dioxygen, can involve a variety of intermediates differing by one in electrons contents :

Superoxide ($:O_2^-$) is the anion of the perhydroxyl radical, HO_2 and has one more electron than dioxygen.

Hydrogen peroxide (H_2O_2) is the undissociated form of the dianion, O_2^{2-}, which has two more electrons than dioxygen.

Hydroxyl radical (·OH) is equivalent to half of a hydrogen peroxide molecule, but is much more reactive. Addition of one electron to each hydroxyl radical results in the formation of hydroxide ions, OH^-, the completely reduced form of oxygen.

Singlet oxygen ($^1O_2$) is a form of dioxygen in which the electrons are in less stable orbitals with antiparallel spins.

Almost without exception, the aerobic cells protect themselves against : O_2^- and H_2O_2 by the action of *superoxide dismutase* and *catalase*, which these cells do contain, respectively. Superoxide dismutase converts superoxide radical into H_2O_2 while catalase transforms H_2O_2 into water and molecular oxygen.

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

Although toxic, H_2O_2 may be useful to some organisms such as **bombardier beetle**. This insect generates a concentrated solution of H_2O_2 in one sac of its spray gland and a solution of hydroquinone

in the other sac. When threatened, the insect frightens (and also poisons) its enemy by firing a hot (100°F) spray of toxic quinone, which is produced from the oxidation of hydroquinone by H_2O_2.

MECHANISMS OF OXIDATIVE PHOSPHORYLATION

One of the most challenging and difficult problems in biochemical research is that how does the electron-transport chain cooperate with the ATP synthetase to bring about oxidative phosphorylation of ADP to ATP? One of the reasons is that the enzymes concerned in electron-transport and oxidative phosphorylation are very complex and they are embedded in the inner mitochondrial membrane, rendering the detailed study of their interactions difficult. However, 3 principal hypotheses have been advanced to account for the coupling of oxidation and phosphorylation. In other words, these hypotheses explain how the energy transfer between electron transport and ATP synthesis takes place.

1. Chemical Coupling Hypothesis

This is the oldest of the 3 hypotheses and proposes that electron transport is coupled to ATP synthesis by a sequence of consecutive reactions in which a high-energy covalent intermediate is formed by electron transport and subsequently is cleaved and donates its energy to make ATP. The hypothesis, thus, postulates direct chemical coupling at all stages of the process. It is similar to the concept in glycolysis which states that the ATP produced in oxidative phosphorylation results from an energy-rich intermediate encountered in electron transport. Specifically, when an oxidoreduction reaction occurs between A_{red} and B_{oxi}, the factor I is incorporated into the formation of an energy-rich structure $A_{oxi} \sim I$, where the ~ indicates a linkage having an energy-rich nature :

$$A_{red} + I + B_{oxi} \rightleftharpoons A_{oxi} \sim I + B_{red}$$

In subsquent reactions, an enzyme (E) replaces A_{oxi} in the compound $A_{oxi} \sim I$ to form an energy-rich $E \sim I$. Later, inorganic phosphate reacts with $E \sim I$ to form phosphoenzyme complex $E \sim P$ containing the energy-rich enzyme-phosphate bond :

$$A_{oxi} \sim I + E \rightleftharpoons A_{oxi} + E \sim I$$
$$E \sim I + Pi \rightleftharpoons E \sim P + I$$

The enzyme-phosphate component finally reacts with ADP to form ATP.

$$E \sim P + ADP \rightleftharpoons E + ATP$$

Although suggestions have been made regarding the nature of $E \sim I$ and $E \sim P$, such compounds have not been identified in mitochondria.

Oxidative phosphorylation occurs in certain reactions of glycolysis, in the citric acid cycle and in the respiratory chain. However, it is only in those phosphorylations occurring at the substrate level in glycolysis and the citric acid cycle that the chemical mechanisms involved are known. Three such equations are given below :

3-phosphoglyceraldehyde + NAD^+ + Pi $\longrightarrow$ 1 ~ 3-biphosphoglycerate + NADH + H^+

1 ~ 3-biphosphoglycerate + ADP $\longrightarrow$ 3-phosphoglycerate + ATP

2-phosphoglycerate $\longrightarrow$ 2 ~ phosphoenolpyurvate

2-phosphoenolpyruvate + ADP $\longrightarrow$ Pyruvate + ATP

α-ketoglutarate + NAD+ + CoA $\longrightarrow$ Succinyl ~ CoA + NADH + H^+

Succinyl ~ CoA + GDP + Pi $\longrightarrow$ Succinate + GTP

Some key differences are evident in these equations. In equation I, phosphate is incorporated into the product of the reaction after the oxidoreduction. In equation II, phosphate is incorporated into the substrate before the internal arrangement or redox change. In equation III, the redox reaction leads to the generation of a high-energy compound other than a phosphate, which in a subsequent reaction leads to the formation of high-energy phosphate.

It is presumed that oxidative phosphorylations in the respiratory chain follow the pattern shown in equations I and 3, the latter being an extension of reaction I, to which an extra nonphosphorylated high-energy intermediate stage is added. Of the possible mechanisms shown in Fig. 23–20, mechanism Ⓒ is favoured since in the presence of uncouplers (see page 452) such as 2, 4-dinitrophenol, oxidoreduction in the respiratory chain is independent of Pi. However, at present, the identities of the hypothetical high-energy carrier (Car ~ I), and the postulated intermediates I and X are not known. In recent years, several so-called "coupling factors" have been isolated that restore phosphorylation when added to disrupted mitochondria.

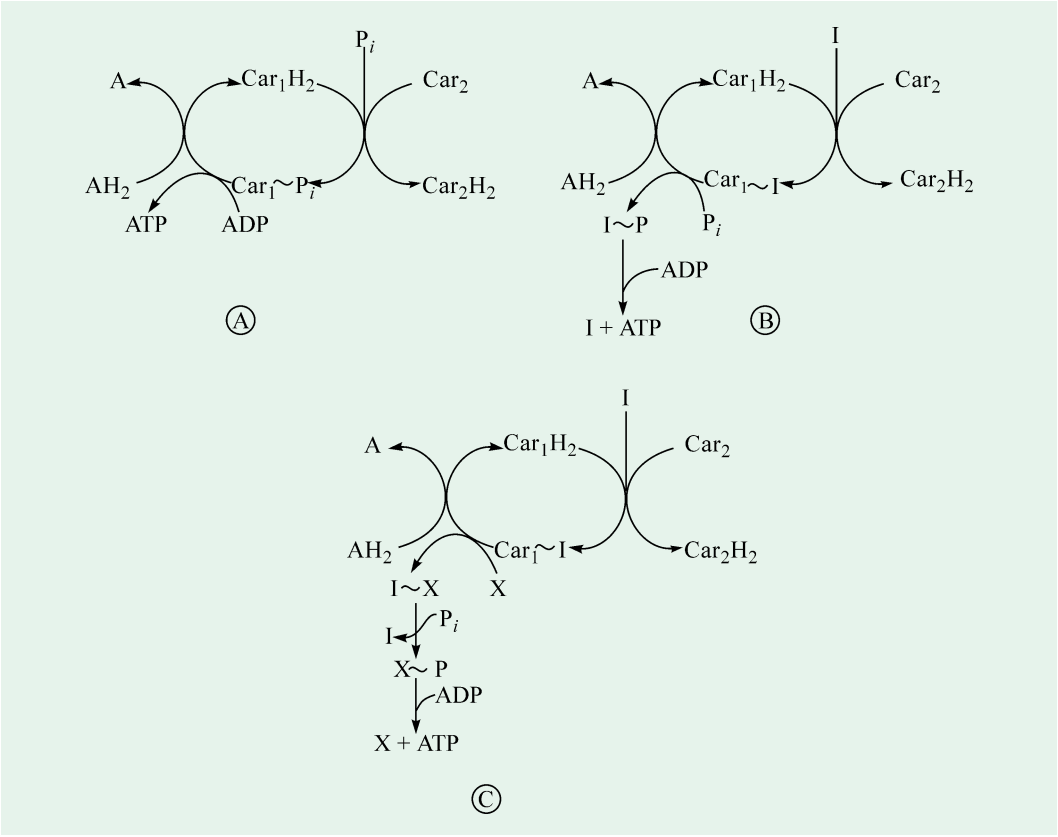
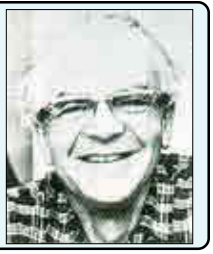


Fig. 23–20. Possible mechanisms for the chemical coupling of oxidation and phosphorylation in the respiratory chain

2. Conformational Coupling Hypothesis

In mitochondria, that are actively phosphorylating in the presence of an excess of ADP, the inner membrane pulls away from the outer membrane and assumes a "*condensed state*". In the absence of ADP, the mitochondria have the normal structure or the "*swollen state*", in which the cristae project into the large matrix. The propounders of this hypothesis believe that the energy released in the transport of electrons along the respiratory chain causes the conformational changes, just described, in the inner mitochondrial membrane and that this energy-rich condensed structure, in turn, is utilized for ATP synthesis as it changes to the energy-poor swollen conformation. However, the mode of the conformational changes that take place in the inner mitochondrial membrane is not yet clearly understood.

Peter Mitchell (LT, 1920 – 1992)



Mitchell, a British biochemist, is a rare example of the truly independent scientist since, in his native England, he was not affiliated with a university, industry or government. He received **1978 Nobel Prize in Chemistry** for his work on the coupling of oxidation and phosphorylation. He proposed that electron transport and ATP synthesis are coupled by a proton gradient, rather than by a covalent high-energy intermediate or an activated protein.

3. Chemiosmotic Coupling Hypothesis

Salient features. This is a simpler radically different and novel mechanism and was postulated by Peter Mitchell, a British biochemist, in 1961. He proposed that *electron trasnsport and ATP synthesis are coupled by a proton gradient, rather than by a covalent high-energy intermediate or an activated protein.* According to this model (Fig. 23.21), the transfer of electrons through the respiratory chain results in the pumping of protons (H^+) from the matrix side (M-side) to the cytosol or cytoplasmic side (C-side) of inner mitochondrial membrane. The concentration of H^+ becomes higher on the

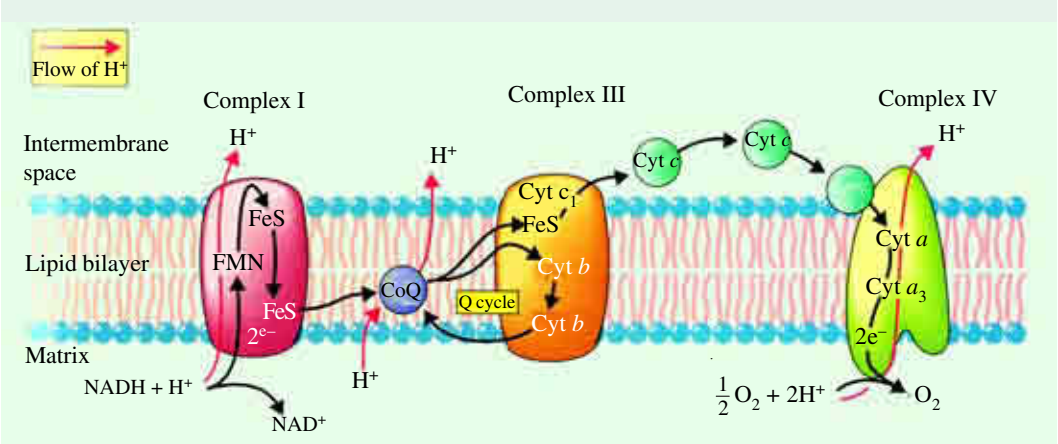


Fig. 23–21. The compositions and locations of respiratory complexes in the inner mitochondrial membrane, showing the flow of electrons from NADH to O_2

Complex II is not involved and not shown. NADH has accepted electrons from substrates such as pyruvate, isocitrate, α-ketoglutarate, and malate. Note that the binding site for NADH is on the matrix side of the membrane. Coenzyme Q is soluble in the lipid bilayer. *Complex III* contains two *b*-type cytochromes, which are involved in the Q cycle. Cytochrome *c* is loosely bound to the membrane, facing the intermembrane space. In *Complex IV,* the binding site for oxygen lies on the side toward the matrix.

The overall effect of the electron transport reaction series is to move protons (H^+) out of the matrix into the intermembrane space, creating a difference in pH across the membrane.

cytoplasmic side, thus creating an electrochemical potential difference. This consists of a chemical potential (difference in pH) and a membrane potential, which becomes positive on the cytoplasmic side. The hypothesis further proposes that the H^+ ions, ejected by electron transport, flow back into the matrix through a specific H^+ channel or 'pore' in the F_oF_1 ATPase molecule, driven by the concentration gradient of H^+. The free energy released, as proton (H^+) flows back through the ATPase, causes the coupled synthesis of ATP from ADP and phosphate by ATP synthetase (Fig. 23–22).

The model requires that the electron carriers in the respiratory chain and the ATP synthetase be anisotropically (= vectorially) organized, i.e., they must be oriented with respect to the two faces of the coupling membrane (= inner mitochondrial membrane). Further, the inner mitochondrial membrane must be intact in the form of a completely closed vesicle (either in intact mitochondria or in submitochondrial vesicles produced during sonication of the inner membrane), since, an H^+ gradient across the inner membrane could not otherwise exist. If, however, a 'leak' of proton across the

membrane is induced by uncouplers, the proton gradient would be discharged and consequently energy-coupling would fail.

Summarily, according to the chemiosmotic hypothesis, the high-energy chemical intermediates are replaced by a link between chemical processes ("chemi") and transport process ("osmotic" – from the *osmos*[G] = push) – hence **chemiosmotic coupling**. As the high-energy electrons from the hydrogens of NADH and $FADH_2$ are transported down the respiratory chain in the mitochondrial inner membrane, the energy released as they pass from one carrier molecule to the next is used to pump protons (H^+) across the inner membrane from the mitochondrial matrix into the innermembrane space. This creates an *electrochemical proton gradient* across the mitochondrial inner membrane, and the backflow of H^+ down this gradient is, in turn, used to drive the membrane-bound enzyme *ATP synthase*, which catalyzes the conversion of oxidative phosphorylation.

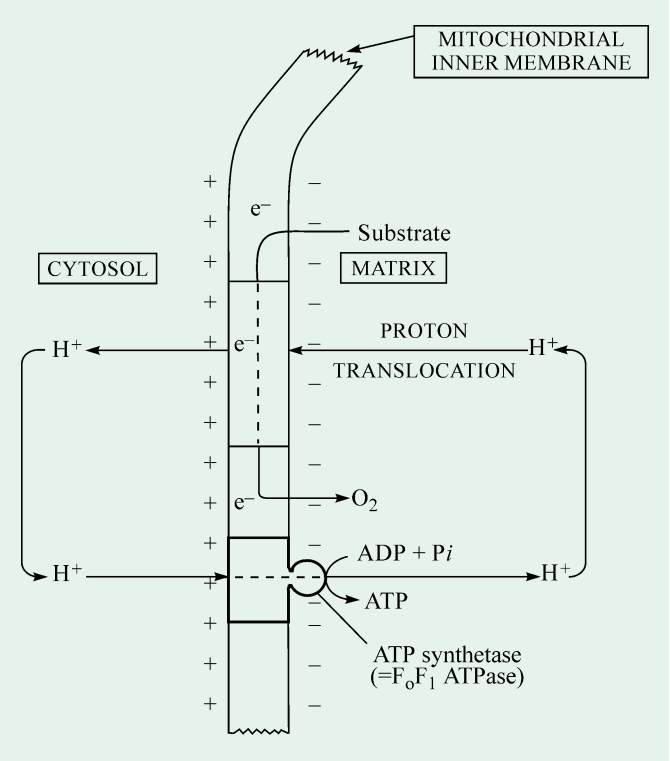


Fig. 23–22. Diagram illustrating principles of the chemiosmotic coupling hypothesis

ATP synthestase (=F_oF_1 ATPase) is responsible for oxidative phosphorylation.

Evidences in Favour. Mitchell's hypothesis that oxidation and phosphorylation are coupled by a proton gradient is supported by a wealth of evidences :

1. No hypothetical 'high-energy' intermediates, linking electron transport to ATP synthesis, have been found to date.
2. Oxidative phosphorylation requires a closed compartment, *i.e.*, the inner mitochondrial membrane should be intact. Breaks or holes in the inner membrane do not allow oxidative phosphorylation, although electron transport from substrates to oxygen may still continue. ATP synthesis coupled to electron transfer does not also occur in soluble cell preparations.
3. The inner mitochondrial membrane is impermeable to H^+, K^+, OH^- and Cl^- ions. If the membrane is damaged in order to pass through such ions readily, oxidative phosphorylation will not take place. However, evidences available indicate the existence of specific transport systems which enable ions to penetrate the inner mitochondrial membrane.
4. Both the respiratory chain and the ATPase are vectorially organized in the coupling membrane.
5. A proton gradient across the mitochondrial inner membrane is generated during electron transport. The pH inside is 1.4 units higher than outside, and the membrane potential is 0.14 V, the outside being positive. The total electrochemical potential Δp (in volts) consists of a membrane potential contribution ($\Delta\psi$) and a H^+ concentration-gradient contribution (ΔpH). Taking R as the gas constant, T as the absolute temperature and F as the caloric equivalent of Faraday, the value of total electrochemical potential (Δp) can be written as :

$$\Delta p = \Delta\psi - \frac{RT}{F} \Delta p\text{H}$$

$$= \Delta\psi - 0.06\ \Delta p\text{H}$$
$$= 0.14 - 0.06\ (-1.4)$$
$$= 0.224\ \text{V}$$

This total proton-motive force of 0.224 V corresponds to a free energy of 5.2 kcal per mole of protons.

6. ATP is synthesized when a *p*H gradient is imposed on mitochondria or chloroplasts in the absence of electron transport.
7. Oxidative phosphorylation can be checked by uncouplers and certain ionophores (see page 453). Uncouplers such as 2,4-dinitrophenol increase the permeability of mitochondria to protons, thus reducing the electrochemical potential and short-circuiting the vectorial ATP synthetase system for the production of ATP.
8. Addition of acid to the external medium, establishing a proton gradient, leads to the synthesis of ATP.

Oxidation-reduction loop. Perhaps the most debated issue of the chemiosmotic hypothesis is the manner in which the process of electron transport in the inner mitochondrial membrane pumps protons (H^+) from the matrix to the exterior. Mitchell has proposed a fantastic scheme which is based on the fact that reducing equivalents are transferred as H atoms by some of the electron carriers (such as ubiquinone), and as electrons by others (such as Fe—S centre and cytochromes). He opined that hydrogen-carrying and electron-carrying proteins alternate in the respiratory chain to form 3 'functional loops' called the *oxidation-reduction loops* (= *o/r loops*). Each loop corresponds functionally to the coupling sites I, II and III of the chemical hypothesis respectively. An ideal single o/r loop consists of a hydrogen carrier and an electron carrier, as depicted in Fig. 23–23. In each loop, two H^+ are carried outward through the inner membrane and deliver two H^+ to the cytosol ; the corresponding pair of electrons is then carried back from the outer to the inner surface of the membrane. Thus, each pair of reducing equivalents passing through such a loop carries two H^+ from the matrix to the exterior. Each loop is thought to provide the osmotic energy to make one mole of ATP.

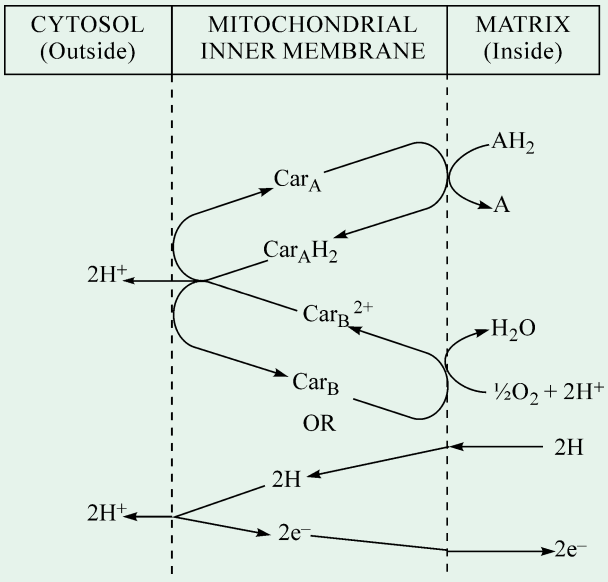


Fig. 23–23. An ideal oxidation-reduction loop as envisaged in chemiosmotic hypothesis

The oxidation-reduction (or o/r) loop translocates protons.

(After Peter A. Mayes, 1979)

Proton Transport Mechanism. In the overall scheme of electron transport, as envisaged by

Peter Mitchell, the respiratory chain is folded into 3 oxidation-reduction (o/r) loops (Fig. 23–24). It is assumed that the members of the respiratory chain are organized in the membrane to provide the necessary sidedness. Each pair of electrons transferred from NADH to oxygen causes 6 protons to be translocated from inside to the outside of the coupling membrane. NADH first donates one H^+ and 2 electrons which, together another H^+ from the internal medium, reduce FMN to $FMNH_2$. FMN extends the full width of the membrane, so that it can release $2H^+$ to the exterior of the membrane and then return 2 electrons to the inside *via* Fe—S proteins which become reduced. Each reduced Fe—S complex donates one electron to ubiquinone (Q) which, after accepting a proton (H^+) from inside the membrane, is reduced to QH_2. QH_2, being a small lipid-soluble molecule, moves to the exterior of the membrane to discharge a pair of protons into the cytosol and donates 2 electrons to 2 moles of cytochrome *b*, the next carrier of electrons in the respiratory chain. Cytochrome *b* is thought to extend the mitochondrial membrane enabling the electrons to join another molecule of ubiquinone along with 2 more protons from the internal medium. The QH_2, so produced, moves to the outer surface to liberate 2 protons and the 2 electrons are passed onto the 2 moles of cytochrome *c*. These electrons, passing through cytochrome *a*, then traverse the membrane to reach cytochrome a_3, which is located on the inner face of the membrane. Here, 2 electrons combine with 2 protons from the internal medium and an oxygen atom to form a molecule of water.

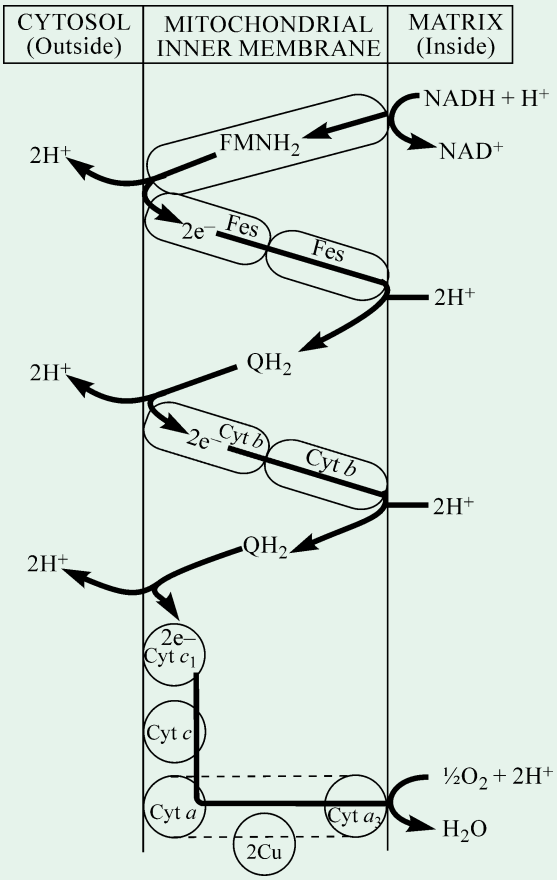


Fig. 23–24. The 'loop' mechanism of proton translocation in the chemiosmotic hypothesis

The 3 o/r loops carry $3 \times 2 = 6H^+$ from the matrix to the cytosol per pair electrons passing from the substrate NADH to oxygen. Much of this mechanism is still tentative, particularly around the Q/cytochrome *b* region.

Inner Membrane Transport Systems. Whereas the mitochondrial outer membrane is freely

permeable to most small solute molecules, the inner membrane is impermeable to H^+, OA^-, K^+ and also many other ionic solutes. How, then, can the ADP^{3-} and HPO_4^{2-} produced in the cytosol enter the matrix and how can the newly-formed ATP^{4-} leave again, since oxidative phosphorylation takes place within the inner matrix space ? Two of the many specific transport systems present in the inner mitochondrial membrane make these events possible (Fig. 23–25) :

1. Adenine nucleotide translocase system. This system consists of a specific protein that extends across the inner membrane. It translocates one molecule of ADP^{3-} inward in exchange for one molecule of ATP^{4-} coming out. As the entrance of ADP is coupled to the exit of ATP, this system is better called as *ADP—ATP antiporter*. Obviously, this transport system is moving more negative charges out than it is bringing in, so it is effectively discharging the outside-positive electrical potential across the inner membrane. The adenine nucleotide transport system is specific since its carries only ATP and ADP and not AMP or any other nucleotides, such as GDP or GTP. Adenine nucleotide translocase is specifically inhibited by a toxic glycoside, *atractyloside*.

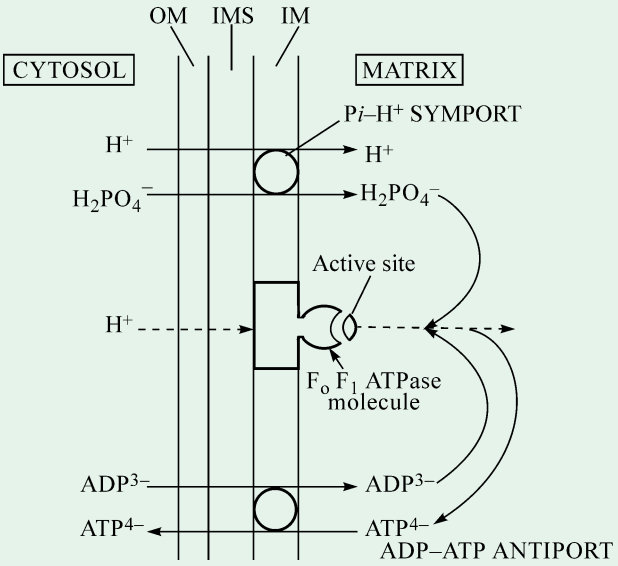


Fig. 23–25. Two principal inner membrane transport systems

[OM = Outer membrane; IMS = Intermembrane space; IM = Inner membrane]

These bring P*i* and ADP into the matrix and allow the newly-synthesized ATP to move out of the matrix.

2. Phosphate translocase system. This is the second membrane system functioning in oxidative phosphorylation. It promotes transport of $H_2PO_4^-$ along with that of H^+ from the cytosol into the matrix compartment. As the entrance of inorganic phosphate is coupled to the entrance of H^+, this system is aptly designated as *Pi—H^+ symporter*. The P*i*–H^+ symporter is electroneutral since it causes no net movement of electrical charge, rather it is effectively transporting protons back into the matrix. The phosphate translocase system is specific for phosphate. It is also inhibited by certain chemical agents.

Thus, the combined action of phosphate and the ADP–ATP translocases allows external phosphate and ADP to enter the matrix and the resulting ATP to return to the cytosol, where most of the ATP-requiring cell activities take place. An **antiporter** exchanges ADP^{3-} and ATP^{4-} and is driven by the electrical potential across the membrane, whereas a **symporter** carries a proton and monobasic P*i* and is driven by the proton concentration gradient. There is no discharge of proton gradient from the release of a proton (H^+) by the transported $H_2PO_4^-$ ion, because there is a counterbalancing uptake when the P*i* is used to make ATP :

$$ADP^{3-} + HPO_4^{2-} + H^+ \longrightarrow ATP^{4-} + H_2O$$

OXIDATION OF EXTRAMITOCHONDRIAL NADH (=NADH Shuttle Systems)

Although NADH cannot penetrate the mitochondrial inner membrane, it is produced continuously in the cytosol by *3-phosphoglyceraldehyde dehydrogenase*, a glycolytic enzyme. However, under aerobic conditions, extramitochondrial NADH does not accumulate and is presumably oxidized by the mitochondrial respiratory chain. How, then, does this occur ? Special shuttle systems have been proposed which are based on the fact that the electrons from cytosolic NADH, rather than cytosolic NADH itself, are carried across the mitochondrial inner membrane by an indirect route. Two shuttle systems, which explain these events, are described below :

1. Malate-oxaloacetate-aspartate shuttle

This shuttle is of comparatively more universal occurrence and operates in heart, liver and kidney mitochondria. This shuttle (Fig. 23–26), is mediated by two membrane carriers and four enzymes. In this shuttle, the reducing electrons are first transferred from cytosolic NADH to cytosolic oxaloacetate to yield malate by the enzymatic action of *cytosolic malate dehydrogenase*. The malate, carrying the electrons, then passes through the inner membrane into the matrix by a dicarboxylate-transport system (A). Here the malate donates its electrons to the matrix NAD^+, reducing it to NADH in the presence of *matrix-malate dehydrogenase*. The NADH then passes its electrons directly to the respiratory chain in the inner membrane. Three moles of ATP are generated as this pair of electrons passes to oxygen. The oxaloacetate, so formed, cannot pass through the mitochondrial inner membrane from the matrix back into the cytosol but is converted by *transaminase* into aspartate which can pass *via* the amino acid-transport system (C). The function of transport system B is to regenerate oxaloacetate into the cytosol. Transport system B makes possible the exchange of glutamate for aspartate. The dicarboxylate-transport system A carries α-ketoglutarate out in exchange for malate passing inward. The net reaction of malate-aspartate shuttle, as it is also called, is :

$$\underset{\text{Cytosolic}}{NADH} + \underset{\text{Mitochondrial}}{NAD^+} \rightleftharpoons \underset{\text{Cytosolic}}{NAD^+} + \underset{\text{Mitochondrial}}{NADH}$$

As evident, this is a readily reversible shuttle, *i.e.*, can operate in both directions either into or out of the mitochondria. The complexity of this system is due to the impermeability of the mitochondrial membrane to oxaloacetate. However, the other anions are not freely permeable and require specific transport systems for passage across the membrane.

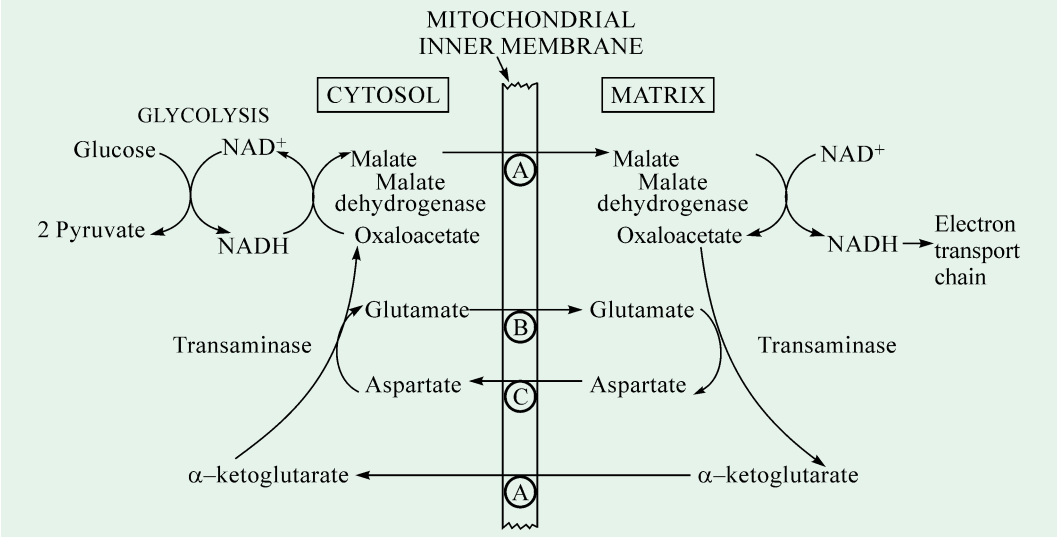


Fig. 23–26. The malate-oxaloacetate-aspartate shuttle

2. Glycerophosphate-dihydroxyacetone phosphate shuttle

This shuttle is not so common and operates prominently in the insect flight muscle and in the brain. This NADH shuttle is medicated by membrane carriers and two enzyme systems, the cytosolic and mitochondrial glycerol 3-phosphate dehydrogenase. *Glycerol 3-phosphate dehydrogenase* is NAD-linked in the cytosol whereas the enzyme found in the matrix is a flavoprotein enzyme. In this shuttle (Fig. 23–27), first of all, the electrons from cytosolic NADH are transferred to cytosolic dihydroxyacetone phosphate (DHAP) to form glycerol 3-phosphate (G-3-P). This reaction is catalyzed by cytosolic glycerol 3-phosphate dehydrogenase. Glycerol 3-phosphate then enters matrix, where it is reoxidized to dihydroxyacetone phosphate by the matrix glycerol 3-phosphate dehydrogenase which is FAD-bound. The DHAP, so formed, then diffuses out of the mitochondria into the cytosol to complete one turn of the shuttle. It is to be noted that as the mitochondrial enzyme is linked to the respiratory chain via a flavoprotein rather than NAD, only 2 rather than 3 mole of ATP are formed per atom of oxygen consumed, as also happens with succinate. The use of FAD enables electrons from cytoplasmic NADH to be transported into mitochondria against an NADH concentration gradient. The price of this transport is one ATP per electron pair. Evidently, if more reducing equivalents are passed through G-3-P—DHAP shuttle, as it is abbreviated, oxygen consumption must increase to maintain ATP production. This mechanism may, therefore, account for at least part of the extra oxygen consumption of hyperthyroid individuals (the administration of thyroxine increases the activity of the flavin-bound glycerol 3-phosphate dehydrogenase). The net reaction of G-3-P—DHAP shuttle is :

$$\underset{\text{Cytosolic}}{NADH} + H^+ + \underset{\text{Mitochondrial}}{\text{E-FAD}} \rightleftharpoons \underset{\text{Cytosolic}}{NAD^+} + \underset{\text{Mitochondrial}}{\text{E-FADH}_2}$$

A remarkable feature of this shuttle is that it is irreversible or unidirectional. *i.e.*, can transfer reducing equivalents only from cytosol to matrix and not *vice versa*.

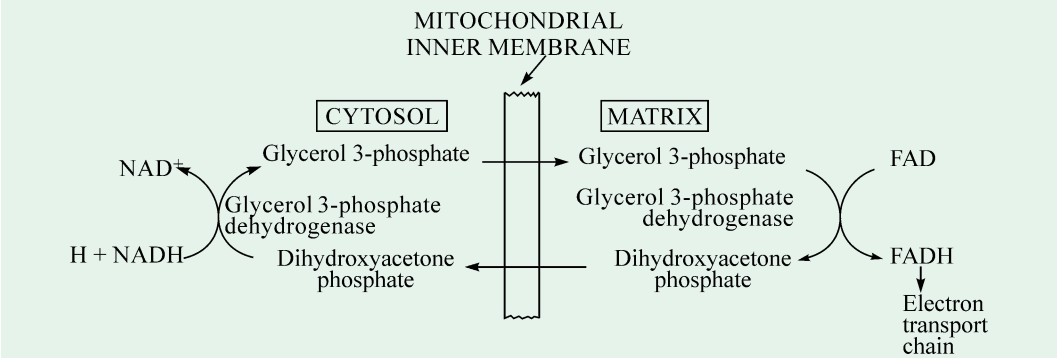


Fig. 23–27. The glycerophosphate-dihydroxyaetone phosphate shuttle

ATP YIELD AND P:O RATIO

We can now calculate step-by-step the recovery of the chemical energy in the form of ATP as a molecule of glucose is completely oxidized to CO_2 and H_2O (Table 23–4) :

1. Firstly, glycolysis of one mole of glucose, under aerobic conditions, yields two moles each of pyruvate, NADH and ATP. The entire process takes place in the cytosol.

   $$\text{Glucose} + 2Pi + 2ADP + 2NAD^+ \longrightarrow 2\text{Pyruvate} + 2ATP + 2NADH + 2H^+ + 2H_2O$$

2. Then, two pair of electrons from the two cytosolic NADH are carried into the mitochondrial matrix through the malate-aspartate shuttle. These electrons next enter the electron-transport chain and flow to oxygen. This produces 2 (3) = 6 ATP, since two NADH are oxidized.

   $$2NADH + 2H^+ + 6Pi + 6ADP + O_2 \longrightarrow 2NAD^+ + 6ATP + 8H_2O$$

   [However, if the glycerophosphate operates in place of malate-aspartate shuttle, only four ATP are generated, instead of six.

   $$2NADH + 2H^+ + 4Pi + 4ADP + O_2 \longrightarrow 2NAD^+ + 4ATP + 6H_2O]$$

3. Then, two moles of pyruvate are dehydrogenated to yield two moles each of acetyl-CoA and CO_2 in the mitochondria. This results in the formation of two NADH. The two electron pairs from two NADH are carried to O_2 via the electron-transport chain, each mole providing three moles of ATP.

   2 Pyruvate + 2CoA + 6P*i* + 6ADP + O_2 ⟶ 2Acetyl-CoA + 2CO_2 + 6ATP + 8H_2O

4. Ultimately, two moles of acetyl-CoA are oxidized to CO_2 and H_2O *via* the citric acid cycle, along with the oxidative phosphorylation coupled to electron transport from isocitrate, α-ketoglutarate and malate to O_2, each of which yields 3 moles of ATP. The oxidation of succinate, however, yields 2 ATP and another two ATPs are generated from succinyl-CoA *via* GTP.

   2 Acetyl-CoA + 24 P*i* + 24 ADP + 4 O_2 ⟶ 2 CoA —SH + 4 CO_2 + 24 ATP + 26 H_2O

Table 23–4. ATP yield from the complete oxidation of glucose

Reaction Sequence	*ATP Yield per Glucose*
Glycolysis (in the cytosol)	
Phosphorylation of glucose	– 1
Phosphorylation of fructose 6-phosphate	– 1
Dephosphorylation of 2 moles of 1, 3-DPG	+ 2
Dephosphorylation of 2 moles of PEP	+ 2
2 NADH are formed in the oxidation of 2 moles of G-3-P	
Conversion of pyruvate into acetyl-CoA (inside mitochondria)	
2 NADH are formed	
Citric acid cycle (inside mitochondria)	
2 moles of GTP are formed from 2 moles of succinyl-CoA	+ 2
6 NADH are formed in the oxidation of 2 moles each of isocitrate, α-ketoglutarate and malate	
2 $FADH_2$ are formed in the oxidation of 2 moles of succinate	
Oxidative phosphorylation (inside mitochondria)	
2 NADH formed in glycolysis ; each yields 2 ATP (assuming transport of NADH by malate-oxaloacetate-aspartate shuttle)	+ 6*
2 NADH formed in oxidative decarboxylation of pyruvate ; each yields 3 ATP	+ 6
2 FADH formed in the citric acid cycle ; each yields 2 ATP	+ 4
6 NADH formed in the citric acid cycle ; each yields 3 ATP	+ 18
NET YIELD PER GLUCOSE	+ 38

* If, however, 2 NADH are transported by the glycerophosphate-dihydroxyacetone phosphate shuttle, there would be an yield of 4 ATP rather than 6. In such a case, the net yield of ATP per glucose oxidized would be 36, instead of 38.

Summing up the 4 equations. On adding the above 4 equations, we get the overall equation for the process of respiration as a whole.

Glucose + 38 P*i* + 38 ADP + 6 O_2 ⟶ 6 CO_2 + 38 ATP + 44 H_2O

Thus, one mole of glucose on complete oxidation to CO_2 and H_2O in the heart, liver and kidney, *where the malate-aspartate shuttle operates*, leads to the production of 38 moles of ATP. The P:O ratio in such cases in 3.16, since 38 ATP are produced and 12 atoms of O_2 are consumed.

The **P : O ratio** is defined as the number of moles of inorganic phosphate incorporated into organic form per atom of oxygen consumed. In other words, the P : O ratio may be taken to mean the number of moles of high-energy phosphate generated per atom of oxygen consumed.

[However, in the skeletal muscles, *where glycerophosphate shuttle operates*, a total of 36 ATP moles is generated per mole of glucose oxidized. In that case, the overall equation is modified to:

$$\text{Glucose} + 36\ P_i + 36\ ADP + 6O_2 \longrightarrow 6\ CO_2 + 36\ ATP + 42\ H_2O$$

Here the P:O ratio is 36/12= 3.0.]

Under standard conditions (1.0 M), the theoretical recovery of free energy in the complete oxidation of glucose is :

$$38\ (7.3/686)\ (100) = 40\%$$

[or 36 (7.3/686) (100) = 38%, when glycerophosphate shuttle operates]. But in the intact cell, the efficiency of this process is high (over 70%) because the cellular concentration of glucose, oxygen, P*i*, ADP and ATP are unequal and much lower than the standard concentration of 1.0 M. The trapping of this amount of energy is a noteworthy achievement for the living cell.

ROLES OF ELECTRON TRANSPORT ENERGY

The main function of electron transport in mitochondria is to provide energy for the synthesis of ATP during oxidative phosphorylation. But the energy generated during electron transport is also used for other biological purposes (Fig. 23–28), which are listed below :

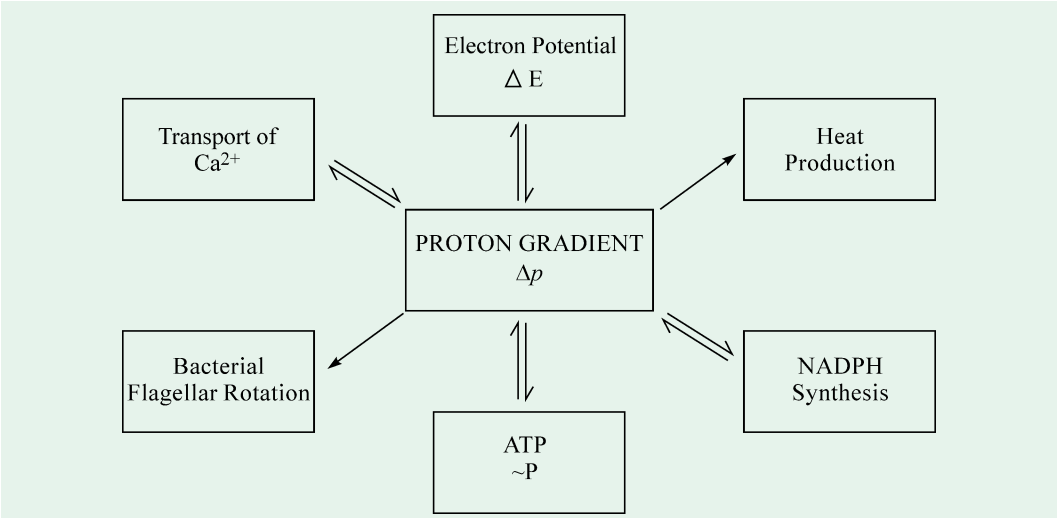


Fig. 23–28. Some key roles of the transmembrane proton gradient

1. The proton gradient generated by electron transport can be used to generate heat. For example, human infants, other mammals born hairless and some hibernating animals have a special type of **brown fat** in the neck and upper back. The brown fat is so named because it contains profuse mitochondria which, in turn, are rich in the red-brown cytochromes. These specialized brown-fat mitochondria do not usually produce ATP, rather they dissipate the free energy of electron transport as heat in order to maintain the body temperature of the young ones. This is because the brown-fat mitochondria have special *proton pores* in their inner membrane that allow the protons, pumped on by electron transport, to flow back into the matrix, rather than through the F_oF_1 ATPase or ATP synthetase. Consequently, the free energy of electron transport is diverted from ATP synthesis into heat production.
2. The electron transport energy is also used to transport Ca^{2+} from the cytosol into the matrix of animal mitochondria. In fact, the inner membrane contains two transport systems for Ca^{2+} : one transports Ca^{2+} inward and the other transports Ca^{2+} outward. The concentration of external Ca^{2+} is maintained at a very low level (about 10^{-7} M). This is due to a balance between the rates of Ca^{2+} influx and efflux. Thus, the inward transport of Ca^{2+} is counterbalanced by Ca^{2+} efflux whose rate is regulated. High Ca^{2+} concentrations initiate or

promote many cell functions such as muscle concentration, glycogen breakdown and the oxidation of pyruvate ; low Ca^{2+} concentrations have inhibitory effects on these functions.

3. Rotation of bacterial flagella is also controlled by the proton gradient generated across the membrane.
4. Transfer fo electrons from NADH to NADPH is also powered by the proton gradient.
5. The entry of some amino acids and sugars is also governed by the energy generated during electron transport.

RESPIRATORY INHIBITORS

The electron transport chain contains 4 complexes (I, II, III and IV) which participate in the transfer of electrons. In general, it may be stated that the structural integrity of these complexes appears essential for its interaction with most inhibitors, since the soluble, phospholipid-free enzymes do not exhibit the characteristic inhibitory pattern. Specific inhibitors are summarized in Table 23–5.

Table 23–5. Different classes of respiratory inhibitors

Class with examples	*Affected complex*or reaction*	*Concentrations employed*
Inhibitors of electron transport		
Rotenone, Piericidin A	I FMN ⟶ Q and /or cyt b (Fe^{3+})	Stoichimetric with f_{PN}
Amytal, Seconal	I same as above	$\geq 10^{-3}$ M
Thenoyltrifluoroacetone	II Peptide-FAD ⟶ Q and/or cyt *b*	$\geq 10^{-4}$ M
Antimycin A, Dimercaprol	III cyt *b* (Fe^{2+}) ⟶ cyt c1 (Fe^{3+}) (perhaps at NH1)	Stoichiometric with cytochromes
HCN, H_2S	IV cyt *c* (Fe^{2+}) ⟶ O_2	$\leq 10^{-4}$ M (CN^-)
HN_3, CO	IV same as above	Higher than above
Inhibitors of oxidative phosphorylation		
Oligomycin Rutamycin Atractylate Bongkrekate	Inhibit respiration (NADH ⟶ O_2 or Succinate O_2) when coupled to phosphorylation	
Uncouplers of oxidative phosphorylation		
2,4 dinitrophenol Dicoumarol Valinomycin † Gramicidin A†	Stimulate respiration when rate is limited by phosphorylation or blocked by oligomycin.	

* The complexes are indicated by Roman numerals.

† Now classed under 'Ionophores of oxidative phosphorylation'.

A. Inhibitors of Electron Transport

(= Inhibitors of Respiratory Chain)

These are the inhibitors that arrest respiration by combining with members of the respiratory chain, rather than with the enzymes that may be involved in coupling respiration with ATP synthesis. They appear to act at 3 loci that may be identical to the energy transfer sites I, II and III (Fig. 23–29).

The various inhibitors of this category are described below :

1. Rotenone. Rotenone (Fig. 23–30) is a compound extracted from the roots of tropical plants such as *Derris elliptica* and *Lonchoncarpus nicou*. It complexes avidly with NADH dehydrogenase and acts between the Fe—S proteins and ubiquinone. Only 30 nanomoles per gram of mitochondrial protein are effective for blocking site I. Rotenone is relatively nontoxic to mammals because it is absorbed poorly, although exposure of the lungs to dust is a little more dangerous. However, the compound is intensely toxic to the fishes and insects as it readily passes into their gills and breathing tubes respectively.

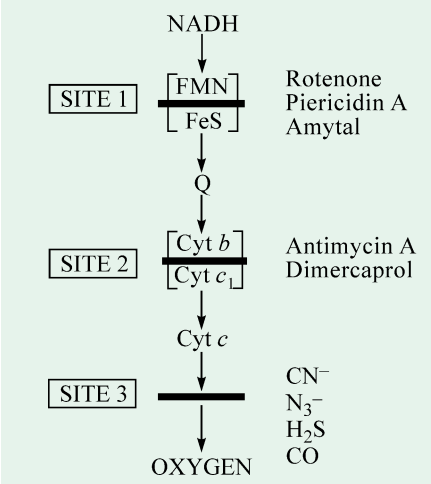


Fig. 23–29. Sites of action of some inhibitors of electron transport

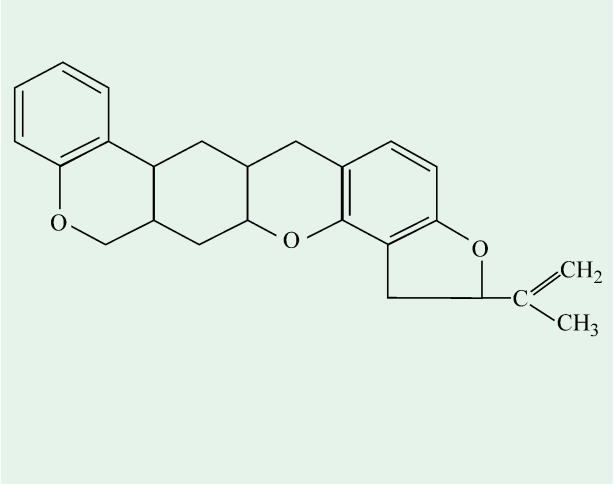


Fig. 23–30. Rotenone

2. Piericidin A. It is an antibiotic, produced by species of *Streptomyces*. It has an action similar to that of rotenone.

3. Barbiturates (Amytal, seconal). These also block NADH dehydrogenase, but are required in much higher concentrations for the purpose. The sedative actions of these compounds appear to depend on their actions on neural membranes, but inhibition of respiration may assist the effect. In contrast, amytal (and also rotenone and piericidin A) do not interfere with the oxidation of succinate, because the electrons of these substrates enter the electron-transport chain beyond the block of coenzyme Q.

4. Antimycins. These are also antibiotics, also produced by *Streptomyces*. They inhibit the respiratory chain at or around site II and block electron flow between cytochromes *b* and c_1, which prevents ATP synthesis coupled to the generation of a proton gradient at site II. This block can be bypassed by the addition of ascorbate, which directly reduces cytochrome *c*. Electrons then flow from cytochrome *c* to O_2, with the concomitant synthesis of ATP coupled to a proton gradient at site III. About 0.07 micromole of antimycin A per gram of mitochondrial protein is effective.

5. Dimercaprol. It is identical in action to the antimycins.

6. Cyanides. These are among the poisons better known by the general public. Although they are not extraordinarily potent (the minimum lethal dose for human beings is between 1 and 3 millimoles), they enter the tissues very rapidly so that a sufficient quantity becomes lethal within a few minutes. It is this quick effect that has gained the cyanides so much respect. The cyanide ion (CN^-) combines tightly with cytochrome oxidase, leading to the cessation of transfer of electrons to oxygen. The previous electron carriers in the chain accumulate in their reduced state, and the generation of high-energy phosphate stops. In fact, the effect of cyanide is as fundamental as deprivation of oxygen, and like the latter causes rapid damage to the brain.

7. Azide. It also blocks the electron flow between the cytochrome oxidase complex and oxygen. Azide (N_3^-), as also cyanides, react with the ferric form (Fe^{3+}) of this carrier.

8. Hydrogen sulfide. Few realize that H_2S is as toxic as HCN. However, its disagreeing odour gives more warning. It is a lethal menace in all drilling operations, especially so on oil-drilling platforms at sea. *In vitro* tests reveal that 0.1 mM sulfide inhibits cytochrome oxidase more than does 0.3 mM cyanide — 96% against 90%.

9. Carbon monoxide. It also attacks between cytochrome oxidase and O_2 but, unlike cyanide and azide, CO inhibits the ferrous form (Fe^{2+}) of the electron carrier.

Phosphorylation coupled to the generation of a proton gradient at site III does not occur in the presence of these inhibitors (*i.e.*, cyanides, azide, H_2S and CO) because electron flow is blocked.

B. Inhibitors of Oxidative Phosphorylation

These compounds inhibit electron transport in cells in which such transport is coupled with ATP synthesis, but they do not affect electron transport in cells in which such transport is not coupled with phosphorylation. Thus, these compounds inhibit both electron transport and oxidative phosphorylation.

1. Oligomycins. These polypeptide antibiotics are obtained from various species of *Streptomyces*. They inhibit the transfer of high-energy phosphate to ADP and, therefore, also inhibit electron transfers coupled to phosphorylation. However, they do not affect those redox reactions that are not coupled. Henceforth, they are widely employed as experimental tools for differentiating between the two kinds of reactions. The Fo component of ATP synthetase binds oligomycin, which is a potent inhibitor of this enzyme and, thus, of oxidative phosphorylation. Apparently, oligomycin appears to block one of the primary phosphorylation steps.

2. Rutamycin. This antibiotic also inhibits both electron transport and oxidative phosphorylation.

3. Atractylate (= Atractyloside). This toxic glycoside is extracted from the rhizomes of *Atractylis gummifera*, a plant native to Italy. It blocks oxidative phosphorylation by competing with ATP and ADP for a site on the ADP—ATP antiport of the inner mitochondrial membrane. Hence, it checks renewal of ATP supply in the cytosol. Evidently, atractylate inhibits at a step beyond that blocked by oligomycin, *i.e.*, one specifically concerned with the entry and exit of ADP/ATP into a mitochondrial compartment.

4. Bongkrekate. It is a toxin formed by a bacteria (*Pseudomonas*) in a coconut preparation (called 'bongkrek') from Java. It also blocks the ADP—ATP antiport. Only 2 micromoles per gram of mitochondrial protein are effective.

C. Uncouplers of Oxidative Phosphorylation

Uncoupling agents are compounds which dissociate (or 'uncouple') the synthesis of ATP from the transport of electrons through the cytochrome system. This means that the electron transport continues to function, leading to oxygen consumption but phosphorylation of ADP is inhibited. In the intact mitochondria, these two processes are intimately associated. When they are uncoupled, the transport of electrons speeds up, thereby pointing out that the phosphorylation of ADP has been a rate limiting process. In the presence of uncouplers, the free energy released by electron transport appears as heat, rather than as newly-made ATP. Uncoupling agents greatly enhance the permeability of the inner membrane to H^+. They are lipophilic and bind H^+ from one side of the membrane and carry it through the membrane toward the side with the lower H^+ concentration. In Mitchell's hypothesis, uncouplers are agents that are capable of destroying the vectorial, anisotropic structure of the membrane, leading to elimination of the pH gradient. As the uncouplers bind and carry protons, they are also called **protonophores**. Uncoupling can be distinguished from inhibition. Uncoupling causes an increased oxygen consumption in the absence of increased utilization of ATP, whereas inhibition of phosphorylation (or inhibition of ADP—ATP antiport) diminishes oxygen consumption in normal coupled mitochondria. Some uncouplers commonly employed are :

1. 2, 4-dinitrophenol (DNP). Introduced by Loomis and Lipmann, dinitrophenol (Fig. 23–31) is one of the most effective agents for uncoupling respiratory-chain phosphorylation. It does not have any effect on the substrate-level phosphorylations that take place in glycolysis. It acts at a concentration of 10 micromolar. The uncoupling action of DNP is due to the fact that both the phenol and the corresponding phenolate ion are significantly soluble in the lipid core of the inner mitochondrial membrane (Fig. 23–32). The phenol diffuses through the core toward the matrix, where it loses a proton ; the phenolate ion then diffuses back toward the cytosol side, where it picks up a proton to repeat the process.

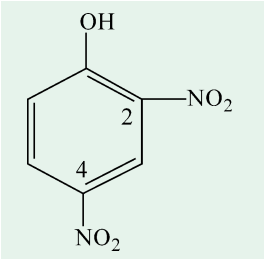


Fig. 23–31. 2,4-dinitrophenol, DNP

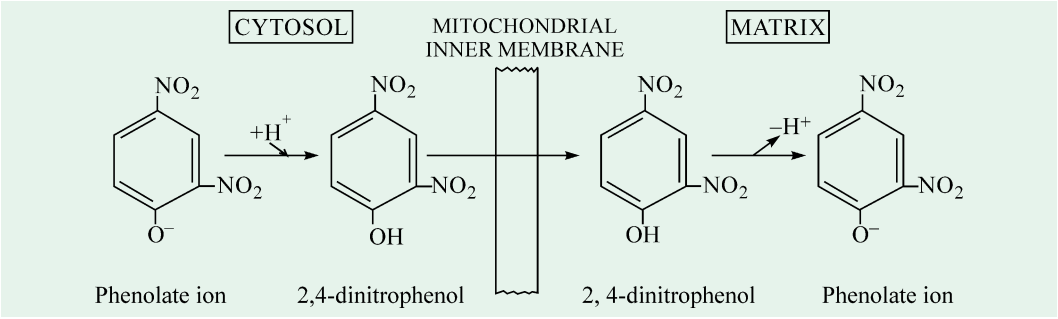


Fig. 23–32. Action of 2,4-dinitrophenol

At *p*H 7.0, this agent exists mainly as the anion which is not soluble in the lipids. In its protonated form, it is lipid-soluble and hence can pass through inner membrane, carrying a proton. The proton (H^+), so carried, is discharged on the other side of the membrane. In this way, uncouplers prevent formation of H^+ gradient across the membrane.

Dinitrophenol also stimulates the activity of the enzyme ATPase, which is normally inactive as a hydrolytic enzyme in mitochondria. Actually, ATP is never formed in the presence of DNP, since the high-energy intermediate is attacked *i.e.*, it acts prior to the step of ATP synthesis.

2. Dicoumarol. Dicoumarol (Fig. 23–33) arises from the action of microorganisms on coumarin, a natural constituent of sweet clover, *Melilotus indica*. It has an action identical to that of 2,4-dinitrophenol. Dicoumarol is also an antagonist of vitamin K function.

Dicoumarol is also spelt as **dicumarol.**

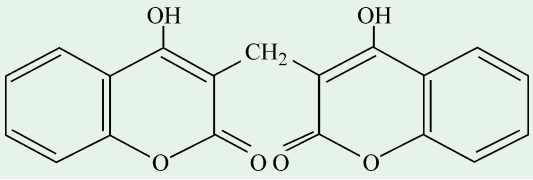


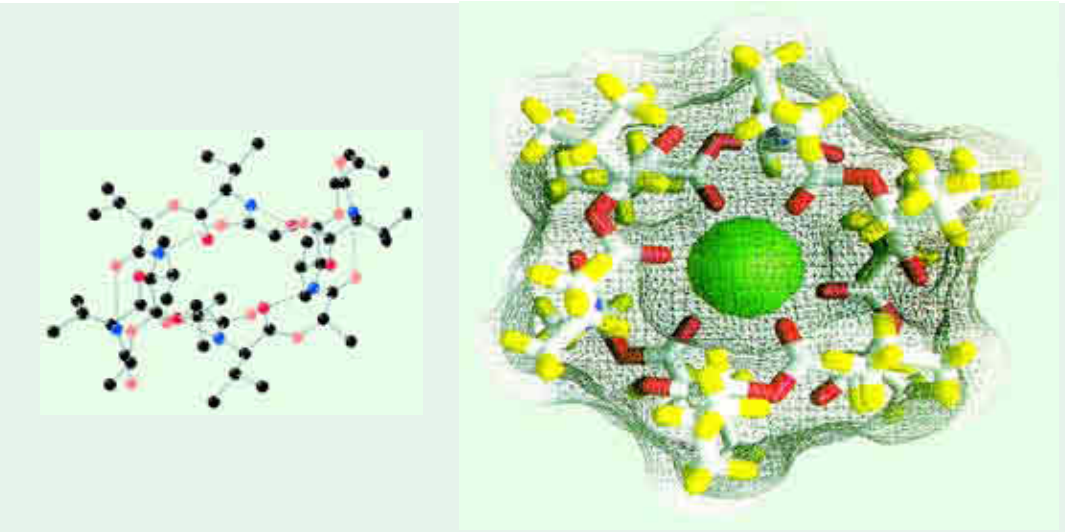
Fig. 23–33. Dicoumarol
(3, 3′-methylene-bishydroxycoumarin)

3. m-chlocarbonyl cyanide phenylhydrazone (CCCP). Its action is also similar to that of 2, 4-dinitrophenol but it is about 100 times more active than the latter.

D. Ionophores of Oxidative Phosphorylation

Ionophores ("ion carriers") are lipophilic substances, capable of binding and carrying specific cations through the biologic membranes. *They differ from the uncouplers in that they promote the transport of cations other than H^+ through the membrane.*

1. Valinomycin. This toxic antibiotic (Fig. 23-34)is synthesized by *Streptomyces*. Valinomycin is a repeating macrocyclic molecule made up of four kinds of residues (L-lactate, L-valine, D-hydroxyisovalerate and D-valine) taken 3 times. The four residues are alternately joined by ester and



(*a*) (*b*)

Fig. 23–34. Valinomycin, a peptide hormone that binds K^+

(*a*) **Ball-and-stick model.** For the sake of clarity, hydrogen atoms are not shown

(*b*) **Computer graphics.** In this image, the surface contours are shown as a transparent mesh through which a stick structure of the peptide and a K^+ atom (*green*) are visible. The oxygen atoms (*red*) that bind K^+ are part of a central hydrophilic cavity. Hydrophobic amino acid side chains (*yellow*) coat the outside of the molecule. Because the exterior of the K^+ valinomycin complex is hydrophobic, the complex readily diffuses through membranes, carrying K^+ down its concentration gradient.

The resulting dissipation of the transmembrane ion gradient kills microbial cells, making valinomycin a potent antibiotic.

(*Courtesy : Smith GD et al, 1975*)

peptide bonds. It contains 6 peptide and 6 ester bonds with side chains consisting of hydrophobic alkyl radicals. The antibiotic forms a lipid-soluble complex with K^+ which readily passes through the inner mitochondrial membrane, whereas K^+ alone in the absence of valinomycin penetrates only very slowly. It has a high degree of selectivity for K^+ as compared to Na^+. In fact, valinomycin binds K^+ about a thousand times as strongly as Na^+ because *water has less attraction for K^+ than for Na^+* and it is energetically more costly to pull Na^+ away from water. Thus, valinomycin interferes with oxidative phosphorylation in mitochondria by making them permeable to K^+. The result is that mitochondria use the energy generated by electron transport to accumulate K^+ rather than to make ATP.

2. Gramicidin A. Gramicidin A (Fig. 23–35) is a linear polypeptide consisting of 15 amino acid residues. Two noteworthy features of the molecule are that (a) the L-and D-amino acids alternate and that (*b*) both the N– and C–terminals of this polypeptide are modified. Gramicidin promotes penetration not only of K^+ but also of Na^+ and several other monovalent cations through the inner membrane. Unlike valinomycin, gramicidins do not complex cations. Rather they induce ion permeability of membranes at concentrations as low as 10^{-10} M by forming dimers which in effect provide `tubes' or 'channels' which span the membrane and through which the cation passes (Fig. 23–36). They are, therefore, better called as 'ion channels' in contrast to valinomycin and allied substances which are classed as 'ion carriers'.

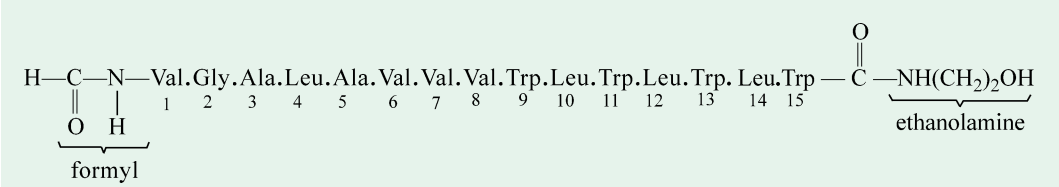


Fig. 23–35. Gramicidin A

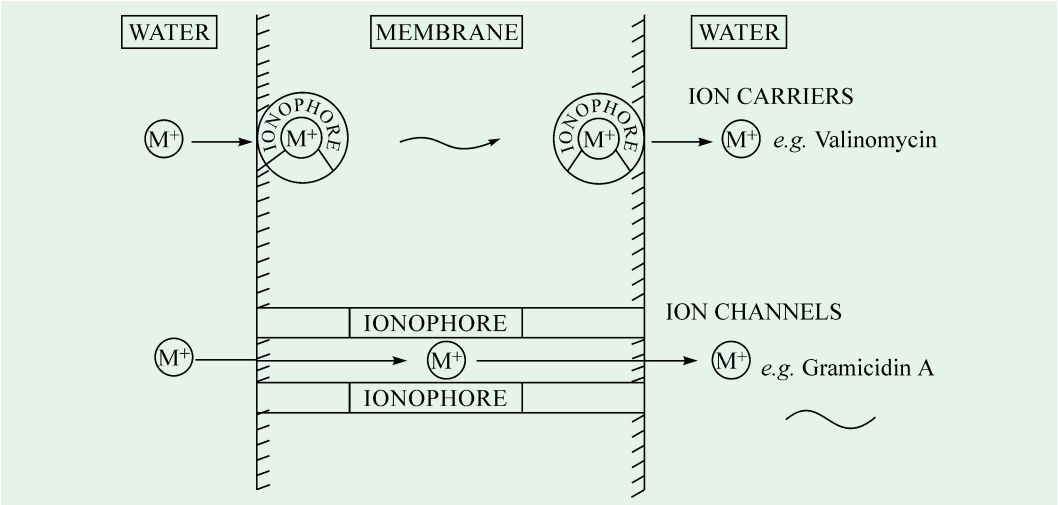


Fig. 23–36. Schematic representationof the action of ionophores on membranes
M + = metal ion

(*After Ovchinnikov YA, 1979*)

3. Nigericin. Like valinomycin, it also acts as an ionophore for K^+ but in exchange for H^+. It, therefore, abolishes the pH gradient across the membrane. In the presence of both valinomycin and nigericin, both the membrane potential and the pH gradient are eliminated, and phosphorylation is, therefore, completely inhibited.

REGULATORY CONTROLS AMONG GLYCOLYSIS, THE CITRIC ACID CYCLE AND OXIDATIVE PHOSPHORYLATION

The 3 energy-yielding stages in carbohydrate metabolism are glycolysis, the citric acid cycle and oxidative phosphorylation. Each stage is so regulated as to satisfy the time-to-time need of the cell for its products. These 3 stages are coordinated with each other in such a way that they function most economically in a self-regulated way. They produce ATP and certian specific intermediates such as pyruvate and citrate, that act as precursors for the biosynthesis of other cell components. The coordination of these 3 stages is brought about by the interlocking regulatory mechanisms (Fig. 23–37). It is apparent from the figure that the relative concentrations of ATP and ADP control not only the rate of electron transport and oxidative phosphorylation but also the rates of glycolysis, pyruvate oxidation and the citric acid cycle.

When ATP concentration is high and ADP and AMP correspondingly low (*i.e.*, the [ATP]/[ADP][P*i*] ratio is high), the rates of glycolysis, pyruvate oxidation, the citric acid cycle and oxidative phosphorylation are at a minimum. But when there is a large increase in the rate of ATP utilization by the cell, with the corresponding increased formation of ADP, AMP and Pi the rate of electron transport and oxidative phosphorylation will immediately increase. Simultaneously, the rate of pyruvate oxidation via the citric acid cycle will increase, thus increasing the flow of electrons into the respiratory chain. These events, in turn, will enhance the rate of glycolysis, thus resulting in an increased rate of pyruvate formation. Thus, *the regulatory controls are both inhibitory and stimulatory.*

Interlocking of glycolysis and the citric acid cycle by citrate augments the action of the adenylate system. Whenever ATP, produced by oxidative phosphorylation, and citrate increase to higher levels, they produce concerted allosteric inhibition of phosphofructokinase (PFK) ; the two together being more inhibitory than the sum of their individual effects. In addition, increased levels of NADH and

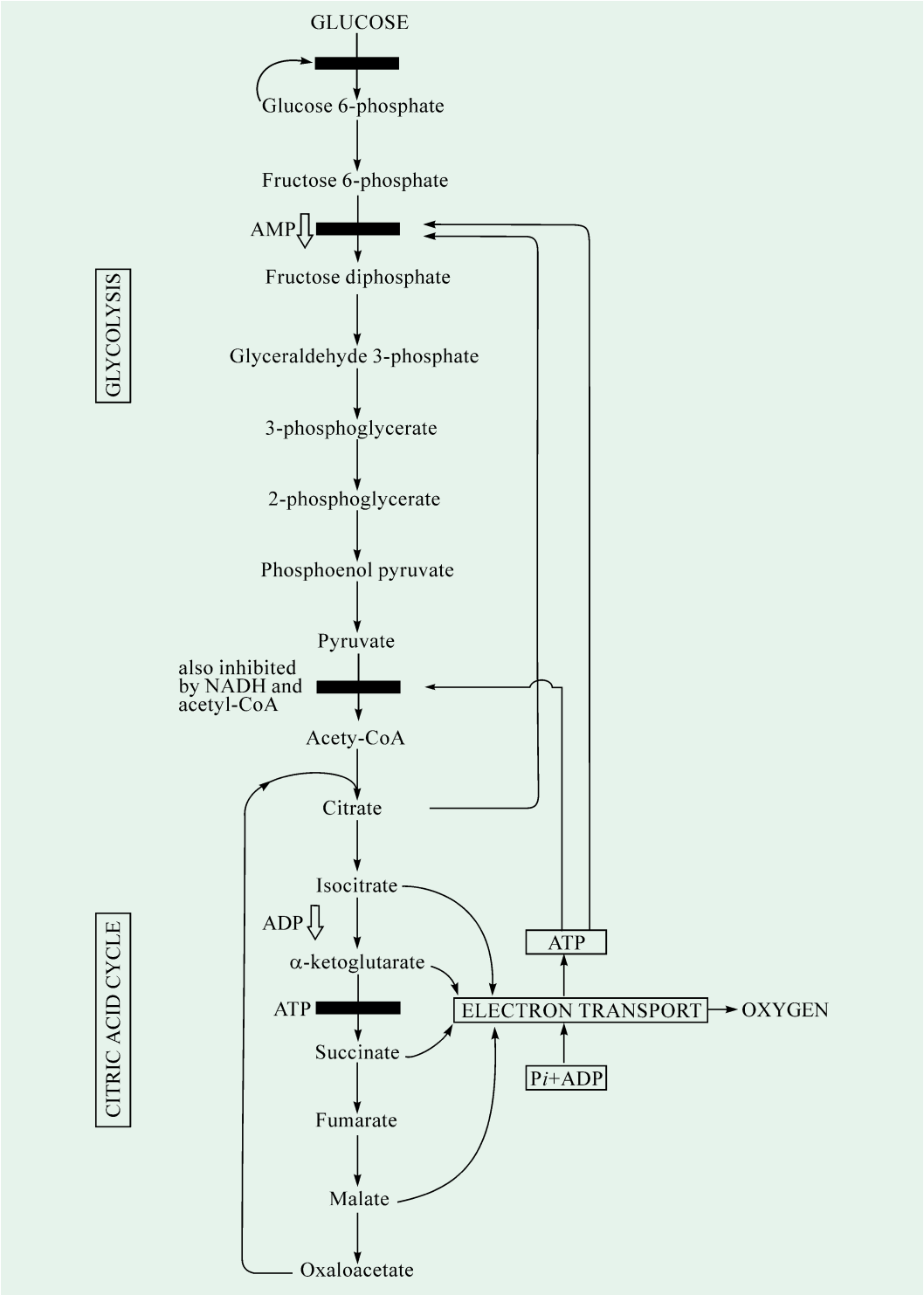


Fig. 23–37. Interlocking regulatory controls among various phases of respiration

The regulatory controls, which are both inhibitory and stimulatory, are shown by solid bars and hollow arrows, respectively.

acetyl-CoA also inhibit the oxidation of pyruvate to acetyl-CoA. In nutshell, interlocking and regulatory mechanisms control glycolysis so that pyruvate is produced at a rate at which it is required by the citric acid cycle, a process which donates electrons for the oxidative phosphorylation.

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PROBLEMS

1. What is the yield of ATP when each of the following substrates is completely oxidized to CO_2 by a mammalian cell homogenate ? Assume that glycolysis, the citric acid cycle, and oxidative phosphorylation are fully active.

(*a*) Pyruvate
(*b*) Lactate
(*c*) Fructose 1, 6-bisphosphate
(*d*) Phosphoenolpyruvate
(*e*) Galactose
(*f*) Dihydroxyacetone phosphate

2. The standard oxidation–reduction potential for the reduction of O_2 to H_2O is given as 0.82 V in Table 23-2. However, the value given in textbooks of chemistry is 1.23 V. Account for this difference.

3. What is the effect of each of the following inhibitors on electron transport and ATP formation by the respiratory chain ?

(*a*) Azide
(*b*) Atractyloside
(*c*) Rotenone
(*d*) DNP
(*e*) Carbon monoxide
(*f*) Antimycin A

4. The number of molecules of inorganic phosphate incorporated into organic form per atom of oxygen consumed, termed the P : O ratio, was frequently used as an index of oxidative phosphorylation.

(*a*) What is the relation of the P : O ratio to the ratio of the number of protons translocated per electron pair ($H^+/2e^-$) and the ratio of the number of protons needed to synthesize ATP and transport it to the cytosol (P/H^+) ?

(*b*) What are the P : O ratios for electrons donated by matrix NADH and by succinate ?

5. The immediate administration of nitrite is a highly effective treatment for cyanide poisoning. What is the basis for the action of this antidote ? (**Hint :** Nitrite oxidizes ferrohemoglobin to ferrihemoglobin.)

6. Suppose that the mitochondria of a patient oxidizes NADH irrespective of whether ADP is present. The P : O ratio for oxidative phosphorylation by these mitochondria is less than normal. Predict the likely symptoms of this disorder.

7. Years ago, it was suggested that uncouplers would make wonderful diet drugs. Explain why this idea was proposed and why it was rejected. Why might the producers of antiperspirants be supportive of the idea ?

8. You are asked to determine whether a chemical is an electron-transport-chain inhibitor or an inhibitor of ATP synthase. Design an experiment to determine this.

9. Years ago there was interest in using uncouplers such as dinitrophenol as weight control agents. Presumably, fat could be oxidized without concomitant ATP synthesis for re-formation of fat or carbohydrate. Why was this a bad idea ?

10. The NADH dehydrogenase complex of the mitochondrial respiratory chain promotes the following series of oxidation–reduction reactions, in which Fe^{3+} and Fe^{2+} represent the iron in iron–sulfur centres, UQ is ubiquinone, UQH_2 is ubiquinol, and E is the enzyme :

(1) $NADH + H^+ + E\text{–}FMN \longrightarrow NAD^+ + E\text{–}FMNH_2$

(2) $\text{E–FMNH}_2 + 2\text{Fe}^{3+} \longrightarrow \text{E–FMN} + 2\text{Fe}^{2+} + 2\text{H}^+$

(3) $2\text{Fe}^{2+} + 2\text{H}^+ + \text{UQ} \longrightarrow 2\text{Fe}^{3+} + \text{UQH}_2$

Sum : $\text{NADH} + \text{H}^+ + \text{UQ} \longrightarrow \text{NAD}^+ + \text{UQH}_2$

For each of the three reactions catalyzed by the NADH dehydrogenase complex, identify (*a*) the electron donor, (*b*) the electron acceptor, (*c*) the conjugate redox pair, (*d*) the reducing agent, and (*e*) the oxidizing agent.

11. The standard reduction potential of any redox couple is defined for the half-cell reaction (or half-reaction) :

Oxidizing agent + *n* electrons $\longrightarrow$ reducing agent

The standard reduction potentials of the NAD^+/NADH and pyruvate/lactate redox pairs are – 0.320 and – 0.185 V, respectively.

(*a*) Which redox pair has the greater tendency to lose electrons ? Explain.

(*b*) Which is the stronger oxidizing agent ? Explain.

(*c*) Beginning with 1 M concentrations of each reactant and product at pH 7, in which direction will the following reaction proceed ?

Pyruvate + NADH + H^+ $\rightleftharpoons$ lactate + NAD^+

(*d*) What is the standard free-energy change, $\Delta G^{\circ\prime}$, at 25 °C for this reaction ?

(*e*) What is the equilibrium constant for this reaction at 25 °C ?

12. Electron transfer functions to translocate protons from the mitochondrial matrix to the external medium to establish a pH gradient across the inner membrane, the outside more acidic than the inside. The tendency of protons to diffuse from the outside into the matrix, where $[\text{H}^+]$ is lower, is the driving force for ATP synthesis *via* the ATP synthase. During oxidative phosphorylation by a suspension of mitochondria in a medium of pH 7.4, the internal pH of the matrix has been measured as 7.7.

(*a*) Calculate $[\text{H}^+]$ in the external medium and in the matrix under these conditions.

(*b*) What is the outside : inside ratio of $[\text{H}^+]$? Comment on the energy inherent in this concentration.

(*c*) Calculate the number of protons in a respiring liver mitochondrion, assuming its inner matrix compartment is a sphere of diameter 15 μm.

(*d*) From these data would you think the pH gradient alone is sufficiently great to generate ATP ?

(*e*) If not, can you suggest how the necessary energy for synthesis of ATP arises ?

13. ATP production in the flight muscles of the fly *Lucilia sericata* results almost exclusively from oxidative phosphorylation. During flight, 187 ml of O_2/h • g of fly body weight is needed to maintain an ATP concentration of 7 μmol/g of flight muscle. Assuming that the flight muscles represent 20% of the weight of the fly, calculate the rate at which the flight-muscle ATP pool turns over. How long would the reservoir of ATP last in the absence of oxidative phosphorylation ? Assume that reducing equivalents are transferred by the glycerol-3-phosphate shuttle and that O_2 is at 25 °C and 101.3 kPa (1 atm). (**Note :** Concentrations are expressed in micromoles per gram of flight muscle.)

14. Iron-containing compounds that act as hydrogen acceptors in the respiratory chain are :

(*a*) flavoproteins

(*b*) dehydrogenases

(*c*) cytochromes

(*d*) oxidases

CONTENTS

Fats provide an efficient means for storing energy for later use.
The processes of fatty acid synthesis (preparation for energy storage) and fatty acid degradation (preparation for energy use) are, in many ways, the reverse of each other. Studies of mice are revealing the interplay between these pathways and the biochemical bases of appetite and weight control.
[*Courtesy* : *Jackson/Visuals Unlimited*]

CHAPTER

24

Oxidation of Fatty Acids

INTRODUCTION

The lipids of metabolic significance in the mammalian organisms include triacylglycerols (= triglycerides, neutral fats), phospholipids and steroids, together with products of their metabolism such as long-chain fatty acids, glycerol and ketone bodies. An overview of their metabolic interrelationships and their relationship to carbohydrate metabolism is depicted in Fig. 24–1.

At least 10 to 20% of the body weight of a normal animal is due to the presence of lipids, a major part of which is in the form of triglycerides which are uncharged esters of glycerol. Body lipids are distributed in varying amounts in all organs and stored in highly specialized connective tissues called *depot*. In these depots, a large part of the cytoplasm of the cell is replaced by droplets of lipids. Body lipids serve as an important source of chemical potential energy.

Fats (or triacylglycerols) are highly concentrated stores of metabolic energy. They are the best heat producers of the three chief classes of foodstuffs. Carbohydrates and proteins each yield 4.1 kilocalories (4.1 kcal) of heat for every gram oxidized in the body ; whereas fats yield 9.3 kcal more than twice as much. The basis of this large difference in caloric yield is that fats contain relatively

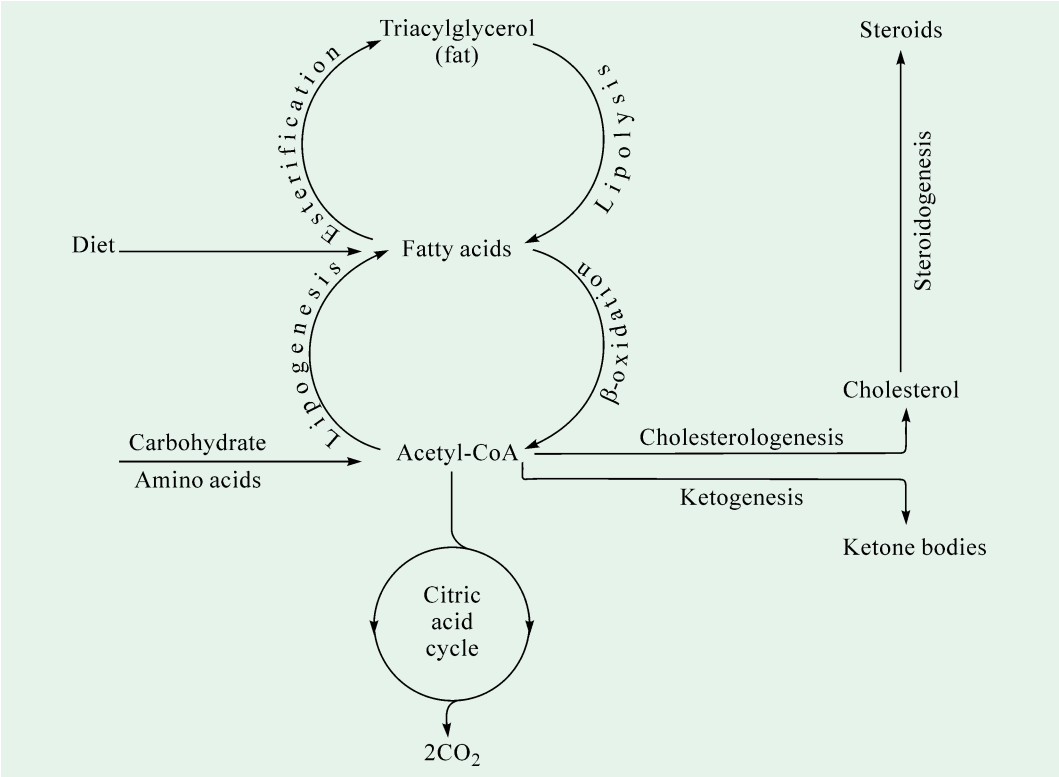


Fig. 24–1. An overview of the principal pathways of lipid metabolism

(*Adapted from Harper, Rodwell and Mayes, 1977*)

more carbon and hydrogen in relation to oxygen as compared to proteins or carbohydrates. In other words, fats are compounds that are less completely oxidized to begin with and therefore can be oxidized further and yield more energy. Furthermore, triacylglycerols are very nonpolar and so they are stored in a nearly anhydrous form, whereas carbohydrates and proteins are much more polar and more highly hydrated. In fact, a gram of dry glycogen (a carbohydrate) binds about 2 grams of water. Consequently, a gram of nearly anhydrous fat stores more than 6 times as much energy as a gram of hydrated glycogen, which is the reason that triacylglycerols, rather than glycogen, were selected in evolution as the major energy reservoir. A normal man, weighing 70 kg, possesses fuel reserves of 10,000 kcal in triacylglycerols, 25,000 kcal in proteins (mostly in muscles), 600 kcal in glycogen and 40 kcal in glucose. As mentioned, triacylglycerols constitute about 11 kg of his total body weight. If this amount of energy were stored in glycogen, his total body weight would be 55 kg greater.

An overview of the intermediary metabolism with special emphasis on fatty acids and triglycerides is given in Fig. 24-2.

The normal animal contains a greater quantity of easily mobilized lipids than of carbohydrates or proteins. About 100 times more energy is stored as mobilizable lipids than as mobilizable carbohydrate in the normal human being. In times of caloric insufficiencies, an animal can meet the endogenous requirements necessary for the maintenance of life by drawing on its lipid depots. In addition, neutral lipids serve as insulators of delicate internal organs of the body. This function is best exemplified in marine animals, whose water environment is both colder than body temperature and a far better thermal conductor than air. Lipids also serve as shock absorbers in protecting joints, nerves and other organs against mechanical trauma.

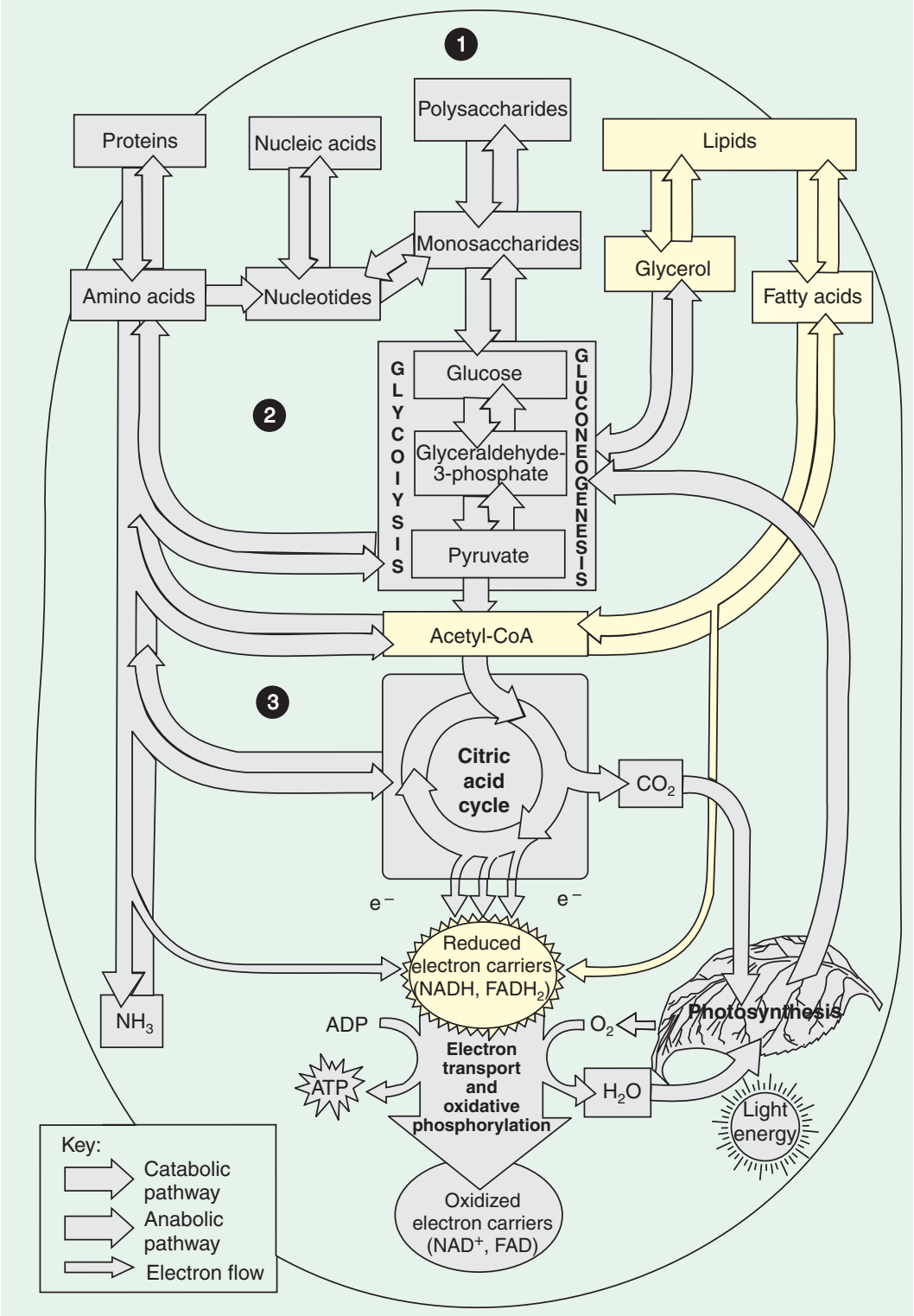


Fig. 24-2. **An overview of intermediary metabolism with fatty acid and triglyceride pathways highlighted**

When food intake exceeds caloric utilization, the excess energy is invariably stored as fat for the body cannot store any other form of food in such large amounts. The capacity of the animal to store carbohydrates (such as glycogen) is strictly limited, and there is no provision for the storage of excess proteins. Moreover, in an adult organism in which active growth has ceased, nitrogen output is more or less geared to nitrogen intake, and the organism shows no tendency to store surplus proteins from the diet. Plants differ from animals in that the energy reserves needed for reproduction are stored in the form of carbohydrates (as in corn or wheat) or as a combination of reserve proteins and oils (as in oil seeds, flax seed, safflower seed or sunflower seed).

In mammals, the major site of accumulation of triacylglycerols is the cytoplasm of adipose cells (= fat cells). Droplets of triacylglycerol coalesce to form a large globule, which may occupy most of the cell volume. Adipose cells are specialized for the synthesis and storage of triacylglycerols and for their mobilization into fuel molecules that are transported to other tissues by the blood. More than 99% of the lipid of human adipose tissue is triacylglycerol, regardless of anatomical location. In general, depot lipid is richer in saturated fatty acids than liver lipid. The more nearly saturated a sample of lipid, the higher the energy yield available from oxidation.

Fig. 24–3 presents, schematically, the flow of lipids in the body. Three important compartments are the liver, blood and adipose tissue. Both liver and adipose tissue are the principal sites of metabolic activity while the blood serves as a transport system. Other compartments, such as cardiac and skeletal muscle, are important utilizers of fatty acids and ketone bodies.

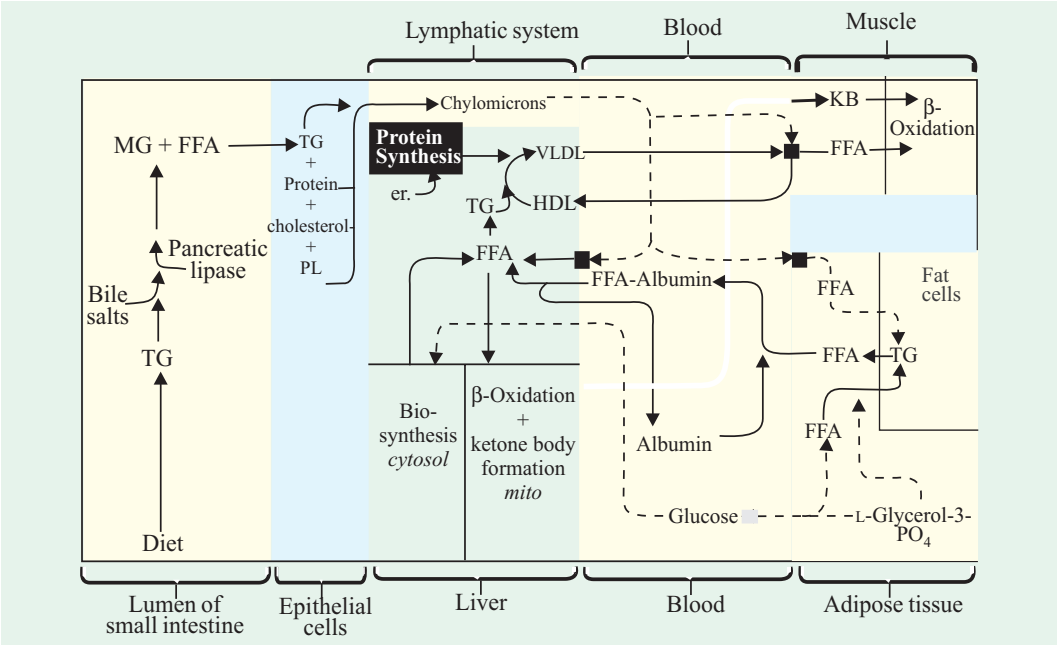


Fig. 24–3. Scheme depicting role of compartments in the utilization of lipids in the animals

TG = triacylglycerol, MG = monoacylglycerol, FFA = free fatty acids, PL = phospholipids, KB = ketone bodies, VLDL = very low-density lipoprotein, HDL = high density lipoprotein, ■ = lipoprotein lipase

(*Adapted from Conn EE, Stumpf PK and Doi RH, 1997*)

A diagrammatic representation of the metabolic interrelationships of fatty acids is presented in fig. 24–4.

OXIDATION OF FATTY ACIDS

General Considerations

The importance of oxidation of fatty acids is not limited to the obese or to devotees of greasy

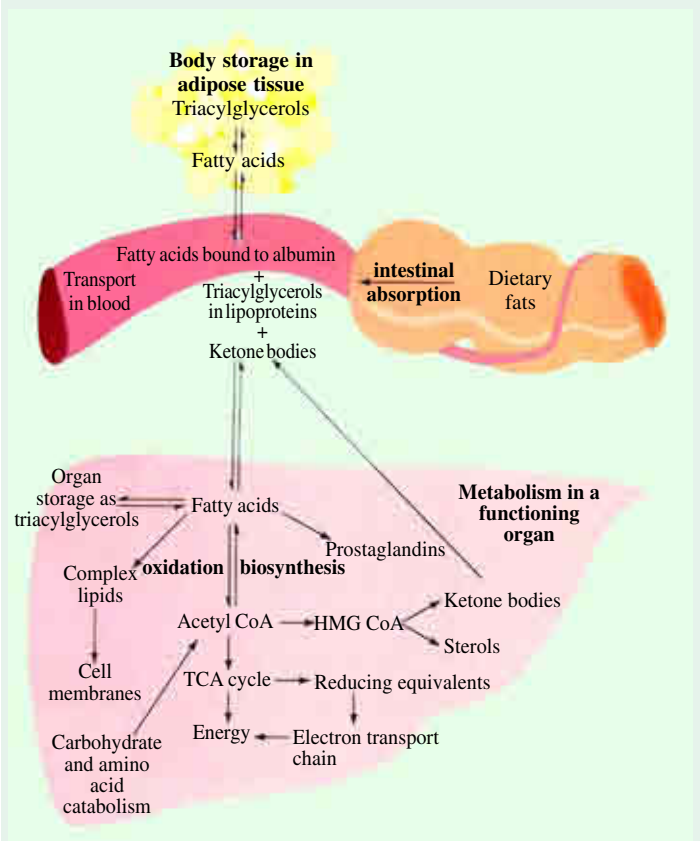


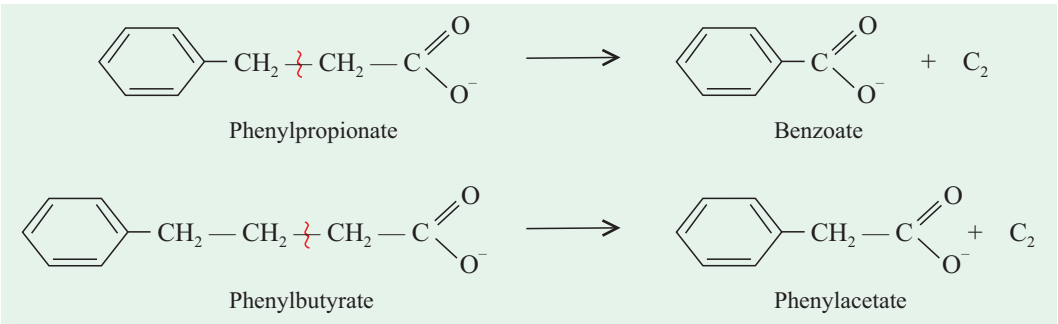
Fig. 24–4. Metabolic interrelationships of fatty acids in the human

foods ; it is a critical part of the metabolic economy in the lean as well as the lardy. The oxidation of long-chain fatty acids to acetyl-CoA is a central energy-yielding pathway in animals, many protists and some bacteria. Complete combustion or oxidaton of a typical fatty acid, palmitic acid, yields 2,380 kcal per mole.

$CH_3—(CH_2)_{14}—COOH + 23\ O_2 \rightarrow\rightarrow\rightarrow\rightarrow 16\ CO_2 + 16\ H_2O + 2380$ kcal/mole

In some organisms, acetyl-CoA produced by fatty acid oxidation has alternative fates. In vertebrate animals, acetyl-CoA may be converted in the liver into ketone bodies, which are water-soluble fuels exported to the brain and other tissues when glucose becomes unavailable. In higher plants, acetyl-CoA from fatty acid oxidation serves primarily as a biosynthetic precursor and only secondarily as fuel. Although the biological role of fatty acid oxidation differs from one organism to another, the mechanism is essentially the same.

In 1904, Franz Knoop elucidated the mechanism of fatty acid oxidation. He fed dogs straight-chain fatty acid in which the ω-carbon atom was joined to a phenyl group. Knoop found that the urine of these dogs contained a derivative of phenylacetic acid when they were fed phyenylbutyrate. In contrast, a derivative of benzoic acid was formed when they were fed phenylpropionate. In fact, benzoic acid was formed whenever a fatty acid containing an odd number of carbon atoms was fed, whereas phenylacetic acid was produced whenever a fatty acid containing an even number of carbon atoms was fed. Knoop deduced from these findings that *fatty acids are degraded by oxidation at the* β*-carbon*. In other words, the fatty acids are degraded in two-carbon units and



that the obvious two-carbon unit is acetic acid. This finding later came to be known as **Knoop's**

hypothesis. These experiments are a landmark in Biochemistry because they were the first to use a synthetic level to elucidate synthetic mechanisms. Deuterium and radioisotopes came into Biochemistry several decades later.

The complete combustion of fatty acids to CO_2 and H_2O occurs in the mitochondria, where the transfer of electrons from the fatty acids to oxygen can be used to generate ATP. The combustion occurs in 2 stages :

(*a*) the fatty acid is sequentially oxidized so as to convert all of its carbons to acetyl-coenzyme A, and

(*b*) the acetyl-coenzyme A is oxidized by the reactions of the citric acid cycle.

Both stages generate ATP by oxidative phosphorylation.

Activation of a Fatty Acid

Because of their hydrophobicity and extreme insolubility in water, triacylglycerols are segregated into lipid droplets, which do not raise osmolarity of the cytosol and, unlike polysaccharides, do not contain extra weight as water of solvation. The relative chemical inertness of triacylglycerols allows their extracellular storage in large quantities without the risk of undesired chemical reactions with other cellular constituents.

But the same properties that make triacylglycerols good storage compounds present problems in their role as fuels. Because of their insolubility in water, the ingested triacylglycerols must be

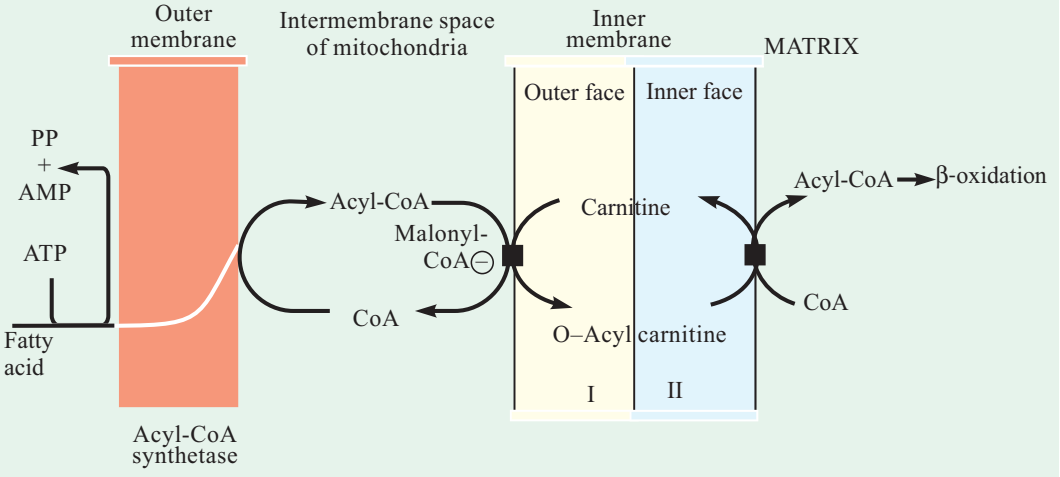


Fig. 24–5. Transport mechanism of fatty acids from cytosol to the β-oxidation site in the mitochondrion

■, Carnitine : acyl-CoA transferase I (outer face) and carnitine : acyl-CoA transferase II (inner face), the two distinct enzymes that catalyze the same reaction; malonyl-CoA ⊖ indicates inhibition of transferase I.

(*Redrawn from Conn EE, Stumpf PK and Doi RH, 1997*)

emulsified before they can be digested by water-soluble enzymes in the intestine, and triacylglycerols absorbed in the intestine must be carried in the blood by proteins that counteract their insolubility. The relative stability of the C—C bonds in a fatty acid is overcome by activation of the carboxyl group at C-1 by attachment to coenzyme A, which allows stepwise oxidation of the fatty acyl group at the C-3 position. This later carbon atom is also called the *beta* (β) carbon in common nomenclature, from which the oxidation of fatty acids gets its common name : β **oxidation.**

An unusual property of liver and other tissue mitochondria is their inability to oxidize fatty acids or fatty acyl-CoA's unless (–)- carnitine (3-hydroxy-4-trimethyl ammonium butyrate) is added in catalytic amounts. Evidently, free fatty acids or fatty acyl-CoA's cannot pentrate the inner membranes of liver and other tissue mitochondria, whereas acyl carnitine readily passes through

the membrane and is then converted to acetyl-CoA in the matrix. Fig. 24–5 outlines the translocation of acetyl-CoA from outside the mitochondrion to the internal site of the β oxidation system. The key enzyme is *carnitine acetyl-CoA transferase*.

Reactions of Fatty Acid Oxidation

The free fatty acids that enter the cytosol from the blood cannot pass directly through the mitochondrial membranes, but must first undergo a series of 3 enzymatic reactions. These are described as under :

First Reaction **:** It is catalyzed by a series of family of isozymes present in the outer mitochondrial membrane, **acyl-CoA synthetases** (also called **fatty acid thiokinases**), which promote the general reaction.

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{Fatty acyl-CoA} + \text{AMP} + \text{PP}i$$
$$\Delta G' = \sim 0 \text{ kcal/mole}$$

Three different acyl-CoA synthetases occur in the cell and act on fatty acids of short, intermediate and long carbon chains, respectively. One type of synthetase activates acetate and propionate to corresponding thioesters, another activates medium chain fatty acids from C_4 to C_{11}, and the third activates fatty acids from C_{10} to C_{20}. Acetyl-CoA synthetase catalyzes the formation of thioester linkage between the fatty acid carboxyl group and the thiol (—SH) group of coenzyme A to yield a fatty acyl-CoA ; simultaneously, ATP undergoes cleavage to AMP and PP*i*. The reaction actually takes place in 2 steps :

$$\text{Fatty acid} + \text{ATP} + \text{Enzyme} \rightleftharpoons \text{Enzyme-acyladenylate} + \text{PP}i$$

$$\text{Enzyme-acyladenylate} + \text{CoA-SH} \rightleftharpoons \text{Enzyme} + \text{Fattyl-acyl-S-CoA} + \text{AMP}$$

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 ($\Delta G^{\circ\prime} \approx -31$ kJ/mol). The formation of fatty acyl-CoAs is made more favourable by the hydrolysis of 2 high energy bonds in ATP; the pyrophosphate formed in the activation reaction is immediately hydrolyzed by a second enzyme, **inorganic pyrophosphatase**, which pulls the preceding activation reaction in the direction of the formation of fatty acyl-CoA. The overall reaction is :

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \longrightarrow \text{Fatty acyl-CoA} + \text{AMP} + 2\ P_i$$
$$\Delta G^{\circ\prime} = -32.5 \text{ kJ/mol}$$

Second Reaction **:** Fatty acyl-CoA esters, formed in the outer mitochondrial membrane, do not cross the inner mitochondrial membrane intact. Instead, the fatty acyl group is transiently attached to the hydroxyl group of carnitine and the fatty acyl-carnitine is carried across the inner mitochondrial membrane by a specific transporter. In this enzymatic reaction, **carnitine acyl-transferase I**, present on the outer face of the inner membrane, catalyzes transesterification of the fatty acyl group from coenzyme A to carnitine. The fatty acyl-carnitine ester crosses the inner mitochondrial membrane into the matrix by facilitated diffusion through the acyl-carnitine/carnitine transporter.

Third Reaction **:** In this final step of the entry process, the fatty acyl group is enzymatically transferred from carnitine to intramitochondrial coenzyme A by **carnitine acyltransferase II.** This isozyme is located on the inner face of the inner mitochondrial membrane, where it regenerates fatty acyl-CoA and releases it, along with free carnitine, into the matrix. Carnitine reenters the space between the inner and outer mitochondrial membranes via the acyl-carnitine/carnitine transporter. Once inside the mitochondrion, the fatty acyl-CoA is ready for the oxidation of its fatty acid component by a set of enzymes in the mitochondrial matrix.

OXIDATION OF EVEN-CHAIN SATURATED FATTY ACIDS (= KNOOP'S β OXIDATION PATHWAY)

Mitochondrial oxidation of fatty acids takes place in 3 stages (Fig. 24–6) :

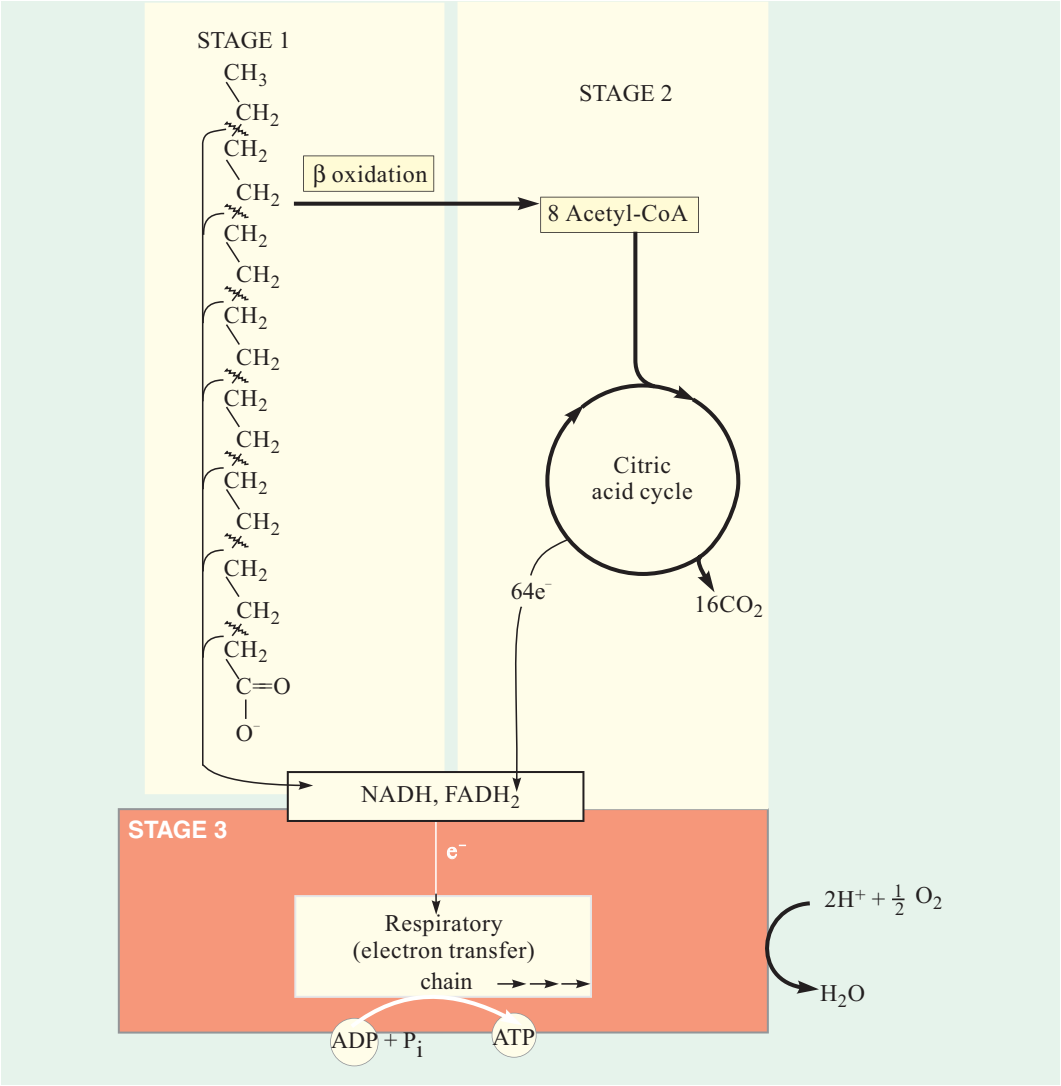


Fig. 24–6. Three stages of fatty acid oxidation

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*)

First Stage : β *oxidation pathway*

In this stage, the 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. For example, the C-16 fatty acid palmitic acid (palmitate at pH 7) undergoes 7 passes through this oxidative sequence, in each pass losing two carbons as acetyl-CoA. At the end of seven cycles, the last two carbons of palmitate (originally C-15 and C-16) are left as acetyl-CoA. The overall result is the conversion of 16-carbon chain of palmitate to 8 two-carbon acetyl-CoA molecules. Formation of each molecule of acetyl-CoA requires removal of 4 hydrogen atoms (two pairs of electrons and 4 H^+) from the fatty acyl moiety by the action of dehydrogenases.

Second Stage : *Citric acid cycle.*

In this stage of fatty acid oxidation, the acetyl residues of acetyl-CoA are oxidized to CO_2 *via* the citric acid cycle, which also takes place in the mitochondrial matrix. Acetyl-CoA derived from fatty acid oxidation, thus, enters a final common pathway of oxidation along with acetyl-CoA derived from glucose *via* glycolysis and pyruvate oxidation.

Third Stage : *Mitochondrial respiratory chain*

The first two stages of fatty acid oxidation produce the electron carriers, NADH and $FADH_2$, which in the third stage donate electrons to the mitochondrial respiratory chain, through which electrons are carried 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 first stage of fatty acid oxidation for the simple case of a saturated chain with an even number of carbons, and for the slightly more complicated cases of unsaturated and odd-number chains, will now be described in detail.

Four Steps of β Oxidation

β Oxidation of saturated fatty acids is accomplished by a 4-step mechanism, illustrated in Fig. 24–7. The four steps of the **fatty acid spiral**, as it is also called, are described below :

First Step : *α, β dehydrogenation of acyl-CoA*

In this step, acyl-CoA is oxidized by an acyl-CoA dehydrogenase to produce an enoyl-CoA with *a trans* double bond between α and β carbon atoms (C-2 and C-3). It is thus, better written as *trans*-Δ^2 -enoyl-CoA (Recall that naturally occurring unsaturated fatty acids normally have their double bonds in the *cis* configuration).

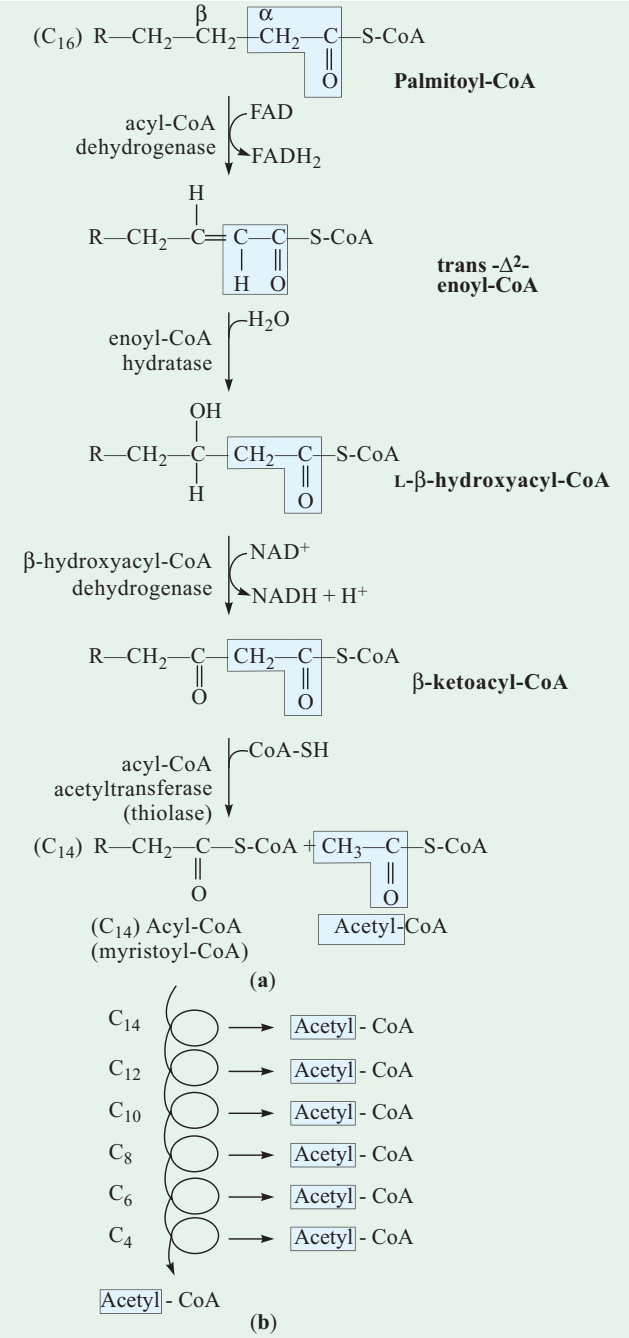
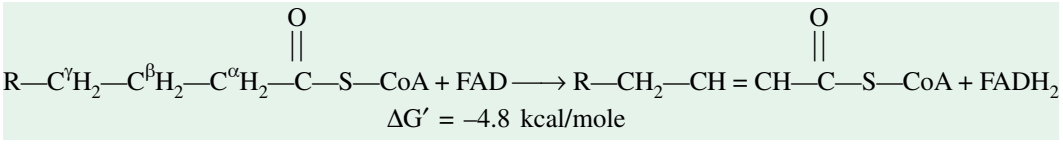


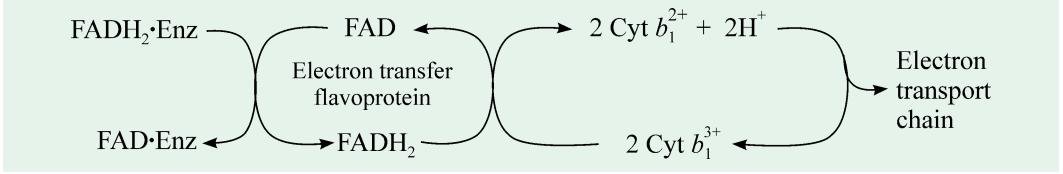
Fig. 24–7. The fatty acid oxidation pathway (= β oxidation cycle)

(a) In each pass through this sequence, one acetyl residue (shaded) is removed in the form of acetyl-CoA from the carboxyl end of palmitate (C_{16}) which enters as palmitoyl-CoA.

(b) Six more passes through the pathway yield 7 more molecules of acetyl-CoA, the seventh arising from the last 2 carbon atoms of the C-16 chain. In all, 8 molecules of acetyl-CoA are formed.



Three *acyl-CoA dehydrogenases* (E.C. No. 1.3.99.3) are found in the matrix of mitochondria. They all have FAD as a prosthetic group. The first has a specificity ranging from C_4 to C_6 acyl-CoAs, the second from C_6 to C_{14} and the third from C_6 to C_{18}. The $FADH_2$ is not directly oxidized by oxygen but traces the following path :



The oxidation catalyzed by acetyl-CoA dehydrogenase is analogous to succinate dehydrogenation in the citric acid cycle (see page 400), as in both the reactions :

(*a*) the enzyme is bound to the inner membrane,

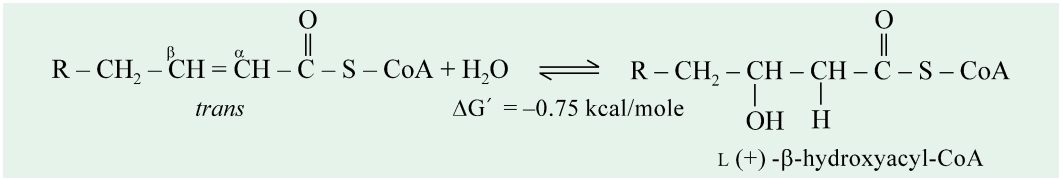
(*b*) a double bond is introduced into a carboxylic acid between the α and β carbons,

(*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 molecules per electron pair.

Second Step : *Hydration of* α, β-*unsaturated acyl-CoAs*

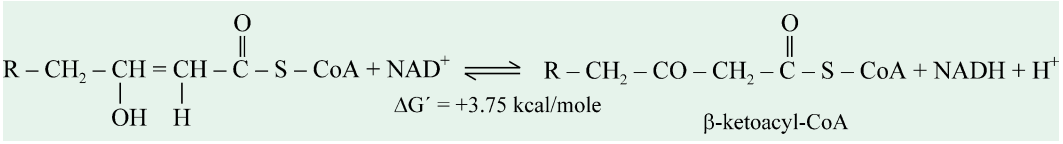
In this step, a mole of water is added to the double bond of the *trans*-Δ^2-enoyl-CoA to form the L-stereoisomer of β-hydroxyacyl-CoA (also called 3-hydroxyacyl-CoA). The reaction is catalyzed by **enoyl-CoA hydratase** or **crotonase** (E.C. No. 4.2.1.17), and has broad specificity with respect to 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 α, β-*cis* unsaturated acyl-CoA, but in this case D (–)-β-hydroxyacyl-CoA is formed).



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 α-β double bond (see page 401). The hydration of enoyl-CoA is, in fact, the prelude to the second oxidation reaction, *i.e.*, Step 3.

Third Step : *Oxidation of* β-*hydroxyacyl-CoA*

In this step of fatty acid oxidation cycle, the L-β-hydroxyacyl-CoA is dehydrogenated (or oxidized) to form β-ketoacyl-CoA by the action of an enzyme, **β-hydroxyacyl-CoA dehydrogenase** (E.C. No. 1.1.1.35), which in 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 (complex I), an electron carrier of the respiratory chain. Three ATP molecules are generated from ADP per pair of electrons passing from NADH to O_2 via the respiratory chain.



This reaction, catalyzed by β-hydroxyacyl-CoA, is closely analogous to the malate dehydrogenase reaction of the citric acid cycle (see page 401). 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 :

Aceyl-CoA ⟶ Enoyl-CoA ⟶ Hydroxyacyl-CoA ⟶ Ketoacyl-CoA

Succinate ⟶ Fumarate ⟶ Malate ⟶ Oxaloacetate

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

Fourth Step : *Thiolysis or Thioclastic scission*

Thiolysis is a splitting by thiol (–SH) group, aided by enzymatic catalysis. This is the final step and brings about the cleavage of β-ketoacyl-CoA by the thiol group of a second mole of CoA, which yields acetyl-CoA and an acyl-CoA, shortened by two carbon atoms. This thiolytic cleavage is catalyzed by the enzyme, **acyl-CoA acetyltransferase** (E.C. No. 2.3.1.16), which also has broad specificity. This enzyme is more commonly called β-**ketothiolase** or simply **thiolase**.

$$R-CH_2-CO-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA + CoA-SH \rightleftharpoons R-CH_2-CO-S-CoA + CH_3-\overset{\overset{O}{\|}}{C}-CoA$$

$\Delta G' = -6.65$ kcal/mole

β-ketoacyl-CoA (n carbons) | Coenzyme A | Acyl-CoA (n-2 carbons) | 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 reactions :

$$R-CH_2-CO-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA + Enz-SH \rightleftharpoons R-CH_2-CO-S-Enz + CH_3-\overset{\overset{O}{\|}}{C}-S-CoA$$

β-ketoacyl-CoA | Thiolase | Acyl-S-Enz | Acetyl-CoA

$$R-CH_2-CO-S-Enz + CoA-SH \rightleftharpoons R-CH_2-CO-S-CoA + Enz-SH$$

Acyl-CoA

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

$$R—CH_2—CH_2—CH_2—CO—S—CoA + FAD + NAD + CoA—SH \longrightarrow R—CH_2—CO—S—CoA + CH_3—CO—S—CoA + FADH_2 + NADH + H^+$$

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 β-oxidation of fatty acids is presented in a cyclical manner in Fig 24-8.

The β oxidative 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 a high lipid content, the β oxidation system is exclusively located in microbodies called glyoxysomes, but in seeds with low lipid content, the enzymes are associated with mitochondria.

Stoichiometry of β Oxidation

The energy yield derived from the oxidation of a fatty acid can be calculated. In each reaction

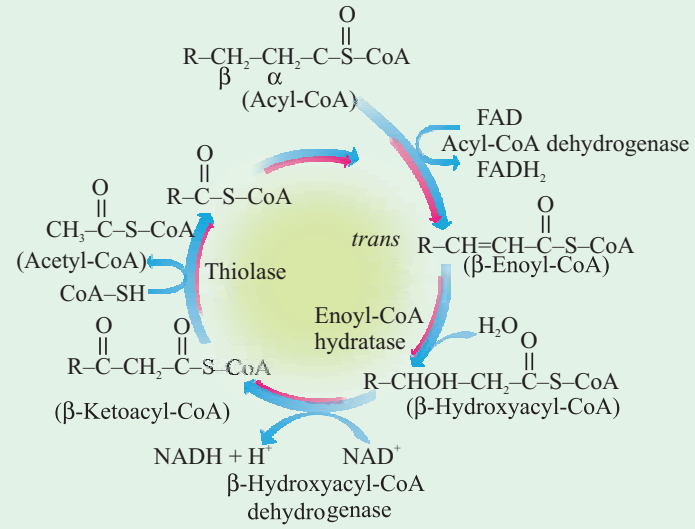


Fig. 24–8. The β-oxidation cycle for fatty acids

cycle, an acyl-CoA is shortened by two carbons and one mole each of $FADH_2$, NADH and acetyl-CoA are formed.

$$C_n\text{–acyl–CoA} + FAD + NAD^+ + CoA + H_2O \longrightarrow C_n\text{–2–acyl–CoA} + FADH_2 + NADH + \text{Acetyl-CoA} + H^+$$

The degradation of palmitoyl-CoA (C_{16}-acyl-CoA), for example, requires 7 reaction cycles. In the seventh cycle, the C_4-ketoacyl-CoA is thiolyzed to 2 moles of acetyl-CoA. Hence, the stoichiometry of oxidation of palmitoyl-CoA is :

$$\text{Palmitoyl-CoA} + 7\ FAD + 7\ NAD^+ + 7\ CoA + 7\ H_2O \longrightarrow 8\ \text{Acetyl-CoA} + 7\ FADH_2 + 7\ NADH + 7\ H^+$$

Three ATP are generated when each of these NADH is oxidized by the respiratory chain, whereas two ATP are formed for each $FADH_2$ because their electrons enter the chain at the level of coenzyme Q. Recall that the oxidation of one mole of acetyl-CoA by the citric acid cycle yields 12 ATP molecules. Hence, the number of ATP moles formed in the oxidation of palmitoyl-CoA is 14 from the 7 $FADH_2$, 21 from the 7 NADH, and 96 from the 8 moles of acetyl-CoA. This totals to 131. Two high-energy phosphate bonds are consumed in the activation of palmitate, in which ATP is split into AMP and 2 P*i*. Thus, *the net yield from the complete oxidation of a mole of palmitate is 131 – 2 = 129 ATP molecules*.

The efficiency of energy conservation in fatty acid oxidation can be estimated from the number of ATP formed and from the free energy of oxidation of palmitic acid to CO_2 and H_2O, as determined by calorimetry. The standard free energy of hydrolysis of 129 ATP is 129×-7.3 kcal = – 941.7 kcal or roughly – 942 kcal. The standard free energy of oxidation of palmitic acid is – 2,340 kcal.

$$CH_3(CH_2)_7\text{—}COOH + 23O_2 \longrightarrow 16CO_2 + 16H_2O$$
$$\Delta G^{\circ\prime} = -2{,}340 \text{ kcal/mole}$$

Hence, the efficiency of energy conservation of the in vivo oxidation of fatty acids, under standard conditions, is $942/2340 \times 100 = 42.56\%$, a surprisingly high figure. This value is similar to those of glycolysis, the citric acid cycle and oxidative phosphorylation. In other words, the 129 ATP produced account for a conservation of 942 kcal of the 2,340 kcal released by the oxidation of one mole of palmitic acid, *i.e.*, roughly 42% efficiency of energy conservation. The remaining energy is lost probably as heat. It, hence, becomes clear why, as a food, fat is an effective source

Fig. 24–9. A grizzly bear preparing its hibernation nest, near the McNeil River in Canada.

of available energy. In this calculation, we neglect the combustion of glycerol, the other component of a triacylglycerol.

In hibernating animals such as grizzly bear (Fig. 24-9) and the tiny dormouse, fatty acid oxidation provides metabolic energy, heat and water — all essential for survival of an animal that neither eats nor drinks for long periods. The camel, although not a hibernator, can synthesize and store triacylglycerols in large amounts in its hump, a metabolic source of both energy and water under desert conditions.

OXIDATION OF UNSATURATED FATTY ACIDS

The fatty acid oxidation scheme described above operates only when the incoming fatty acid is a saturated one (having only single bonds) and possesses 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_2O to the *trans* double bond of the Δ^2 - enoyl-CoA generated during β oxidation. However, by the action of two auxiliary enzymes, the fatty acid oxidation sequence described above can also break down the common unsaturated fatty acids. The action of these two enzymes, one an isomerase and the other a reductase, will be illustrated by the following two examples :

(*a*) Oxidation of Monounsaturated Fatty Acids

This 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*-Δ^9) is aken as an example (Fig. 24–10). 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 Δ^3, 12-carbon unsaturated fatty acid, *cis*-Δ^3- dodecenoyl-CoA (Fig. 24–10). 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*-Δ^3-enoyl-CoA is isomerized to yield the *trans*-Δ^2-enoyl-CoA. The latter compound is now converted by enoyl-CoA hydratase into the corresponding L-β-hydroxyacyl-CoA (*trans*-Δ^2-dodecenoyl-CoA). This intermediate is now acted upon by the remaining enzymes of β oxidation to yield acetyl-CoA and a 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 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*-Δ^9, *cis*-Δ^{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*-Δ^3, *cis*-Δ^6 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. 24–11) allows reentry of

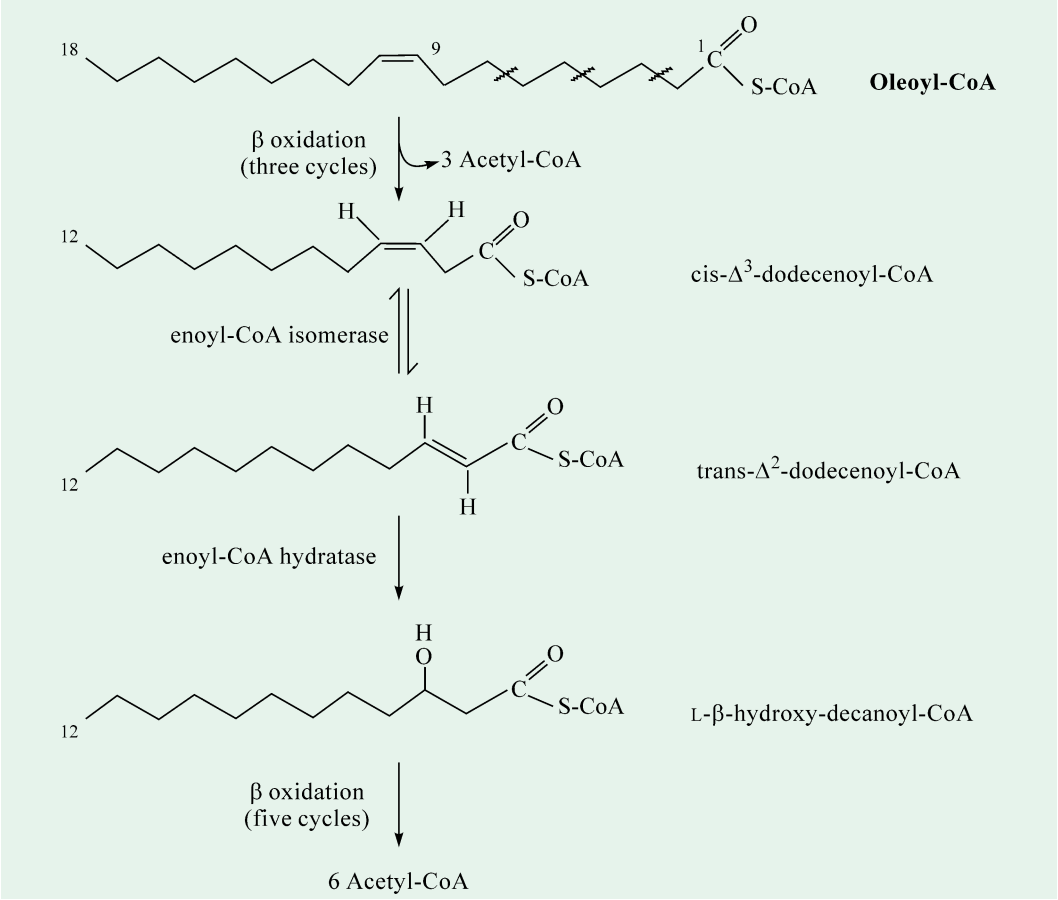


Fig. 24–10. The oxidation of a monounsaturated fatty acyl-CoA such as oleoyl-CoA (Δ9) requiring an additional enzyme, enoyl-CoA isomerase

Note that the enzyme repositions the double bond converting the *cis* isomer to a *trans* isomer, a normal intermediate in β oxidation. Thus, both position and configuration of the double bond are shifted by the action of the enzyme.

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 excellent example of the beautiful economy of organization of metabolism.The introduction of 2 additional types of enzymes (an enoyl-CoA isomerase and a 3-hydroxyacyl-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 below, 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.

$$C_{18:2}\ (9\ cis,\ 12\ cis) \xrightarrow{-3C_2} C_{12:2}\ (3\ cis,\ 6\ cis) \xrightarrow{A} C_{12:2}\ (2\ trans,\ 6\ cis)$$

$$\xrightarrow{-C_2} C_{10:1}\ (4\ cis) \xrightarrow{-C_2} C_{8:1}\ (2\ cis) \xrightarrow{B} \text{3-D-hydroxy}\ C_{8:0}$$

$$\xrightarrow{C} \text{3-L-hydroxy}\ C_{8:0} \xrightarrow{-C_2} C_{6:0} \longrightarrow 3C_2$$

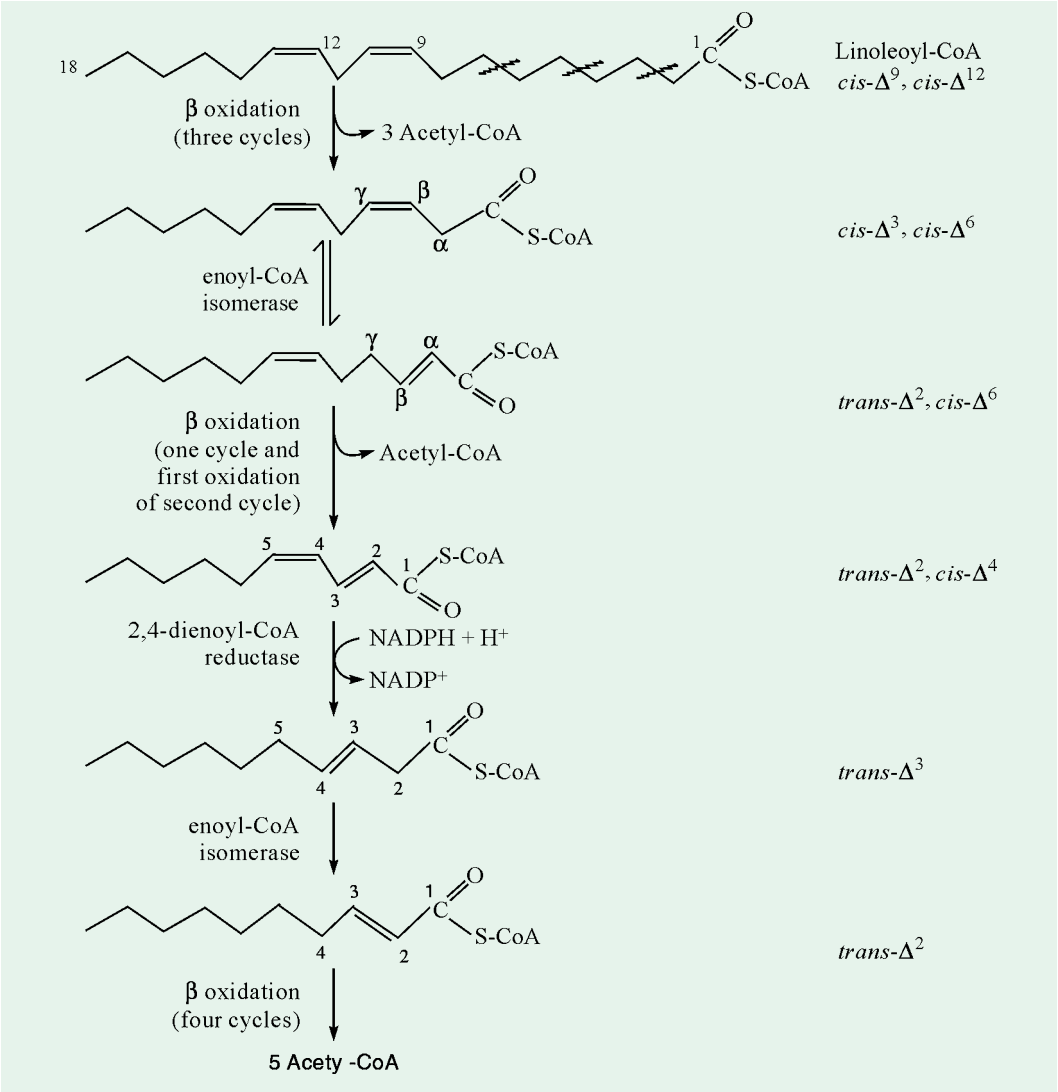


Fig. 24–11. The oxidation of polyunsaturated fatty acids requiring two additional enzymes, enoyl-CoA isomerase and 2, 4-dienoyl-CoA reductase

Note that the combined action of these two enzymes converts a *trans*-Δ^2, *cis* Δ^4-dienoyl-CoA intermediate into the *trans*-Δ^2-enoyl-CoA substrate, necessary for β oxidation.

OXIDATION OF ODD-CHAIN FATTY ACIDS

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 inhibitor to some breads and cereals, and thus propionate enters the human diet. Besides, cattle and other ruminants form large amounts of propionate during fermentation of carbohydrates in the rumen. The propionate so formed is absorbed into the blood and oxidized by the liver and other tissues.

A generalized scheme of the oxidation of an odd-chain fatty acid is presented in Fig. 24-12.

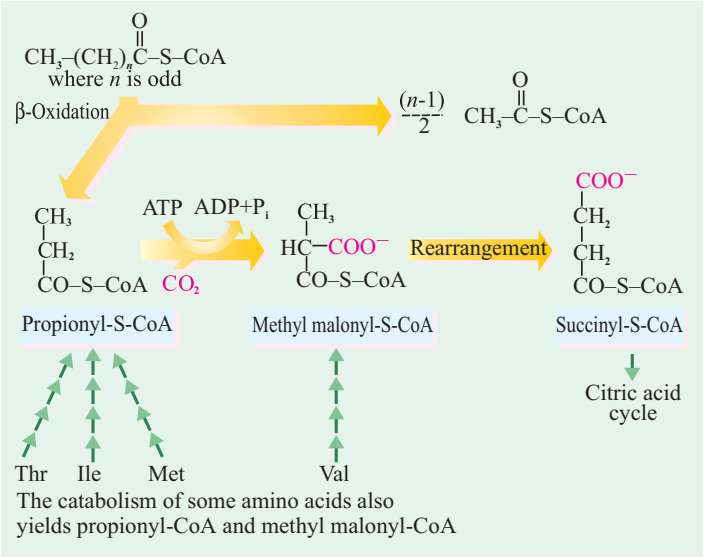


Fig. 24–12. The oxidation of a fatty acid containing an odd number of carbon atoms

The odd-carbon long-chain fatty acids are oxidized by the same pathway as the even-carbon fattty 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.

(*a*) Methylmalonate Pathway

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

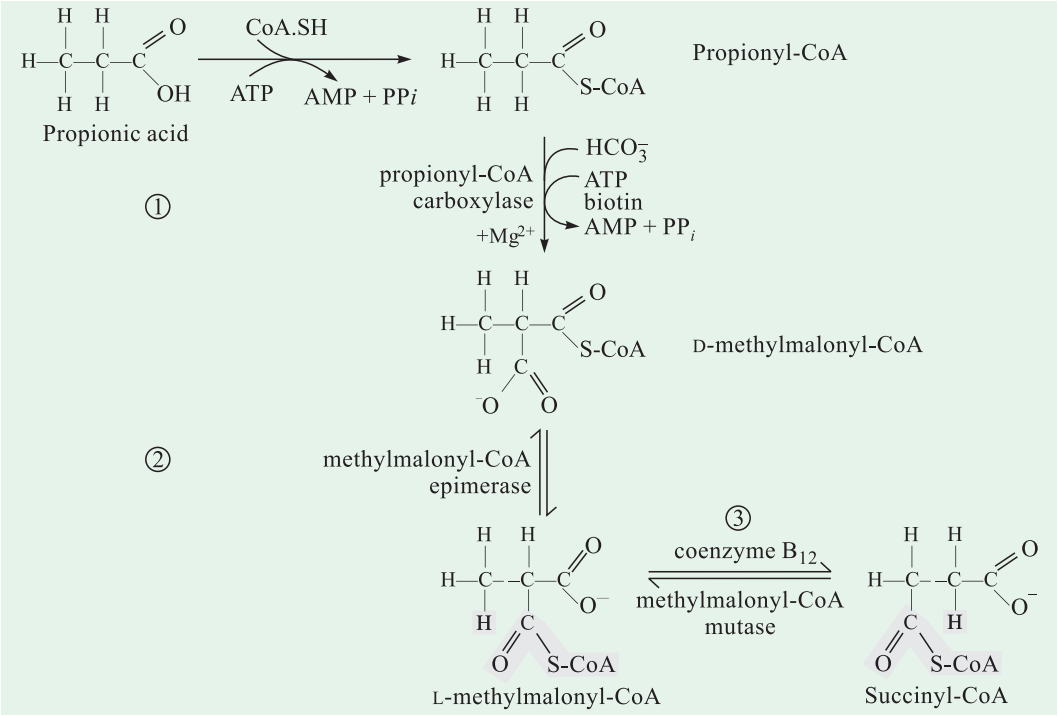


Fig. 24–13. The methylmalonate pathway of propionate metabolism, as found in animals

Note the third remarkable reaction in which substituents on adjacent carbon atoms exchange positions ; the coenzyme B_{12} playing a key role in it.

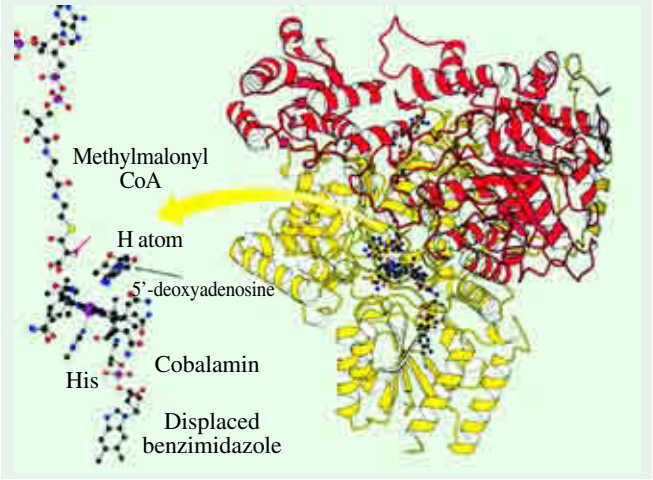
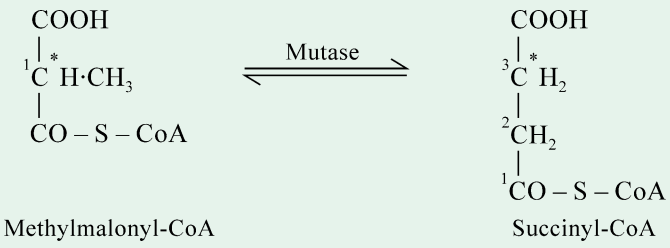
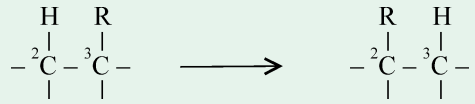


Fig. 24–14. Active site of methylmalonyl CoA mutase
The arrangement of substrate and coenzyme in the active site facilitates the cleavage of the cobalt-carbon bond and the subsequent abstraction of a hydrogen atom from the substrate.

oxidation of isoleucine, valine, methionine and threonine. Propionate is catalyzed by acetyl-CoA synthetase to produce propionyl-CoA (Fig. 24–13). The propionyl-CoA is carboxylated to form the D stereoisomer of methylmalonyl-CoA by an enzyme propionyl-CoA carboxylase, which contains the cofactor biotin. In this reaction, as in pyruvate carboxylase reaction (see page 413), 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 PP*i*. The d-methylmalonyl-CoA, thus formed, is enzymatically epimerized to L-methylmalonyl-CoA, by the action of methylmalonyl-CoA epimerase (The epimerase labilizes the α-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 (Fig 24–14), which requires as its coenzyme deoxyadenosyl-cobalamin or coenzyme B_{12}. When [2–^{14}C] methyl-malonyl-CoA was converted by the mutase enzyme, the label (marked by an asterisk, below) 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 a hydrogen from one carbon atom by transferring it directly to an adjacent carbon atom, simultaneously effecting the exchange of a second (R) substituent. The H and R are not released into solution.



At equilibrium, formation of succinyl-CoA favoured by a ratio of 20 : 1 over methylmalonyl-CoA. The succinyl-CoA can then be oxidized via succinate and the citric acid cycle to CO_2 and H_2O. 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 retardness. 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 CO_2, even in the absence of propionyl-CoA carboxylase.

(b) β-hydroxypropionate Pathway

This pathway is ubiquitous in plants and is a modified form of β 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 enzymes, the methylmalonate pathway does not operate in them. This pathway (Fig. 24–15), thus, bypasses the B_{12} barrier in an effective way.

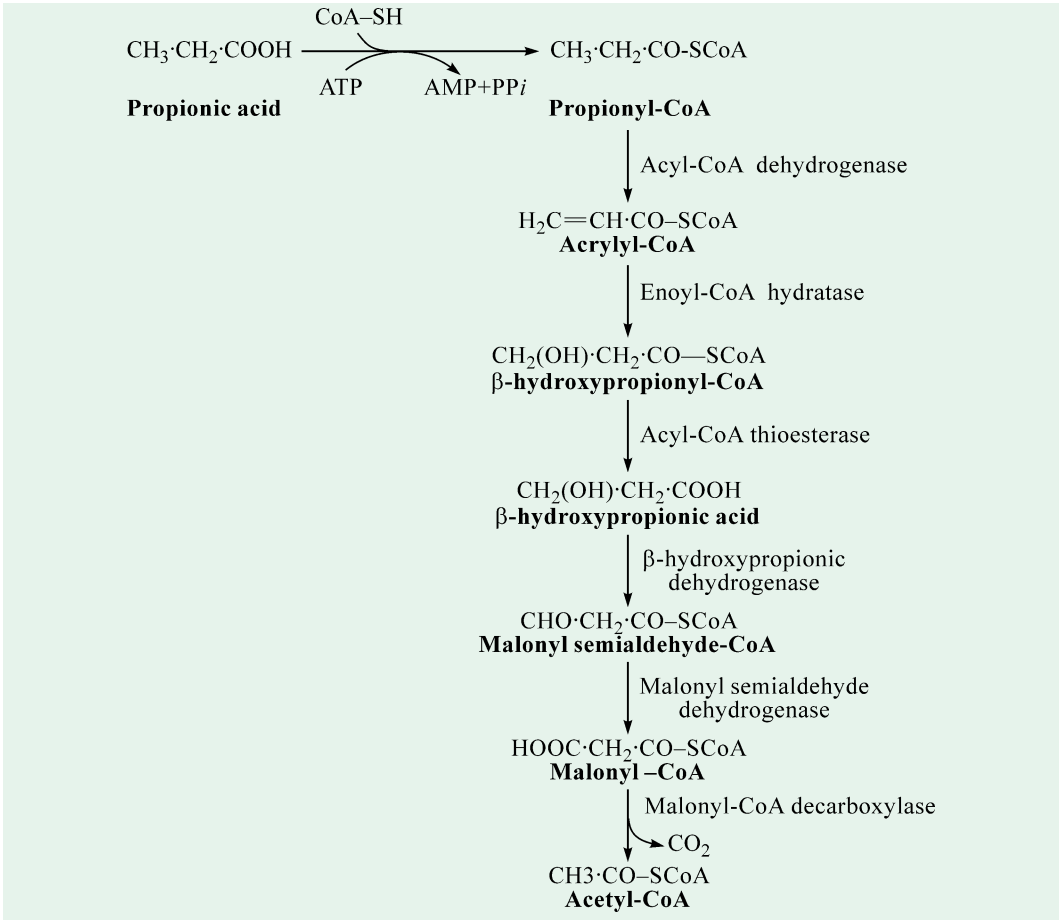


Fig. 24–15. The β-hydroxypropionate pathway of propionate metabolism, as found in plants

α OXIDATION OF FATTY ACIDS

Although β oxidation is major pathway for the oxidation of fatty acids, two other types of oxidation also occur, α and ω oxidation. α oxidation is the removal of one carbon atom (*i.e.*, α

carbon) at a time from the carboxyl end of the molecule. α oxidation was first observed in seeds and leaf tissues of plants. α oxidation of long-chain fatty acids to 2-hydroxy acids and then to fatty acids with one carbon atom less than the original substrate have been demonstrated in the microsomes of brain and other tissues also. Long-chain α hydroxy fatty acids are constituents of brain lipids, *e.g.*, the C_{24} cerebronic acid (= 2 hydroxylignoceric acid), $CH_3\,(CH_2)_{21}.\,CH(OH).\,COOH$. These hydroxy fatty acids can be converted to the 2-keto acids, followed by oxidative decarboxylation, resulting in the formation of long-chain fatty acids with an odd number of carbon atoms :

$$\underset{\substack{\text{Long-chain fatty acid}\\(n\text{ carbon})}}{R{\cdot}CH_2\,CH_2\,CH_2\,COOH} \xrightarrow[\text{Monoxygenase}]{\text{Hydroxylation}} \underset{\text{2-hydroxy fatty acid}}{R{\cdot}CH_2\,CH_2\,CHOH{-}COOH} \longrightarrow$$

$$\underset{\text{2-keto fatty acid}}{R{\cdot}CH_2\,CH_2\,CO{-}COOH} \xrightarrow{\substack{\text{Oxidative}\\\text{decarboxylation}}} \underset{\substack{\text{Long-chain fatty acid}\\(n-1\text{ carbon})}}{R{\cdot}CH_2\,CH_2\,COOH} + CO_2$$

The initial hydroxylation reaction is catalyzed by a mitochondrial enzyme, monoxygenase that requires O_2, Mg^{2+}, NADPH and a heat-stable cofactor. Conversion of the α hydroxy fatty acid to CO_2 and the next lower unsubstituted acid appears to occur in the endoplasmic reticulum and to require O_2, Fe^{2+} and ascorbate.

The salient features of α oxidation are as follows :

1. Only free long-chain fatty acids serve as substrates.
2. Molecular oxygen is indirectly involved.
3. It does not require CoA intermediates.
4. It does not lead to generation of high-energy phosphates.

This mechanism explains the occurrence of α hydroxy fatty acids and of odd-numbered fatty acids in the biomolecules. The latter may, in nature, also be synthesized *de novo* from propionate.

The α oxidation system plays a key role in the capacity of mammalian tissues to oxidize **phytanic acid** (= 3,7,11,15-tetramethylhexadecanate). Phytanic acid is an oxidation product of phytol and is present in animal fat, cow's milk and foods derived from milk. The phytol presumably originates from plant sources, as it is a substituent of chlorophyll and the side chain of vitamin K_2.

Normally, phytanic acid is rarely found in serum lipids because of the ability of normal tissue to degrade (or oxidize) the acid very rapidly. But large amounts of phytanic acid accumulate (as much as 20% of the serum fatty acids and 50% of the hepatic fatty acids) in the tissues and serum of individuals with **Refsum's disease**, a rare inheritable autosomal recessive disorder affecting the nervous system because of an inability to oxidize this acid. Diets low in animal fat and milk products appear to relieve some of the symptoms of Refsum's disease.

The presence of 3-methyl group in phytanic acid (Fig. 24–16) blocks β oxidation. In the mitochondria of normal individuals, α hydroxylation of phytanic acid by *phytanate α hydroxylase* is followed by oxidation by phytanate α oxidase to yield CO_2 and pristanic acid (= 2,6,10, 14-tetramethylpentadecanoic acid), which readily undergoes β oxidation after conversion to its CoA derivative. *In Refsum's disease, there is a lack of the enzyme, phytanate α hydroxylase.*

ω OXIDATION OF FATTY ACIDS

The biological oxidation of fatty acids at the omega (ω) carbon atom was first reported by Verkade and his group, who isolated from the urine dicarboxylic acids of the same chain length as those that were fed in the form of triglycerides. He proposed that certain acids were first oxidized at the ω carbon atom and then further metabolized by β oxidation proceeding from both ends of the dicarboxylic acid.

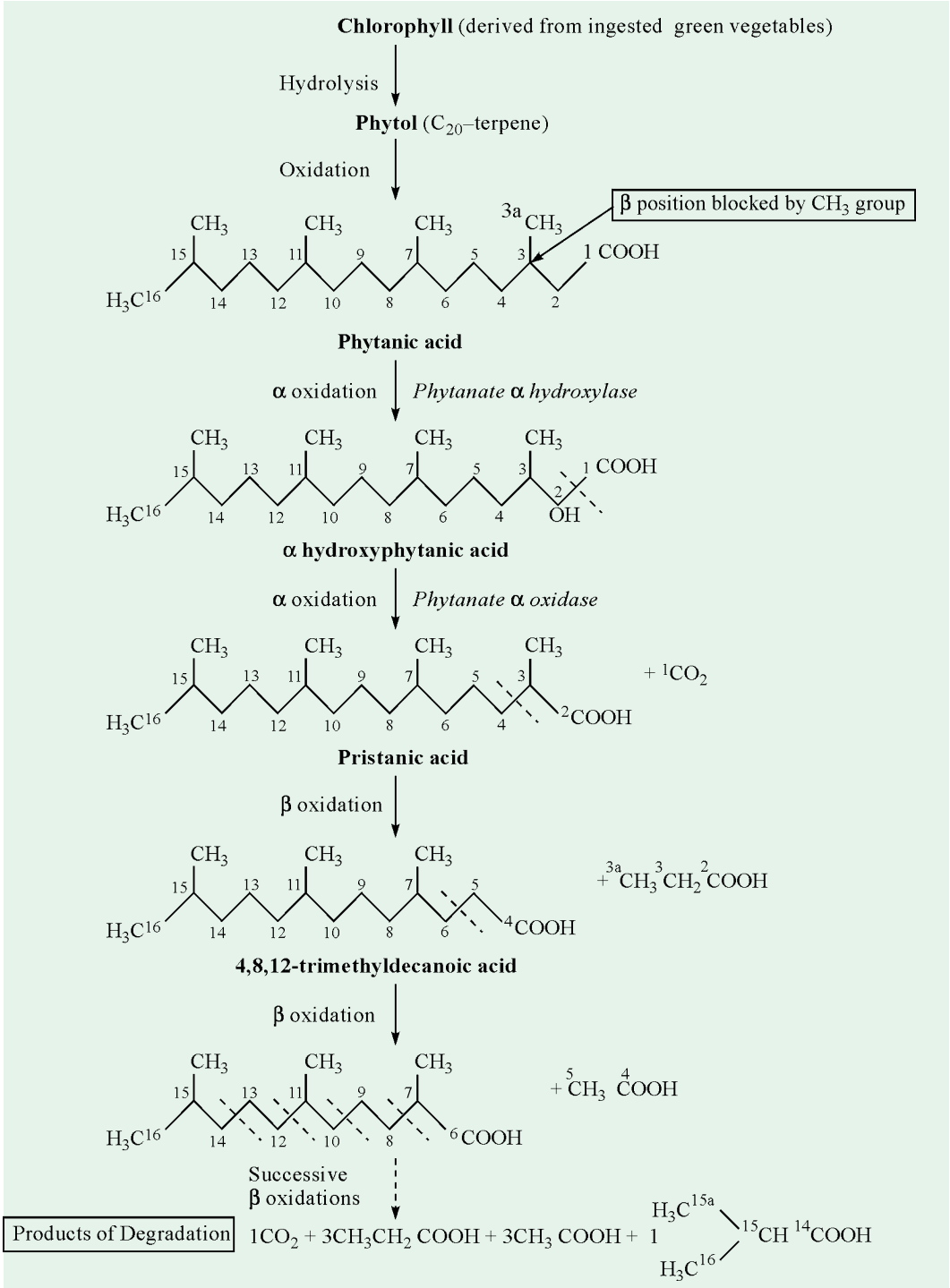


Fig. 24–16. The metabolism of phytanic acid by the normal animal cell
Dashed lines show the successive points of cleavage of the molecule.

The ω oxidation scheme responsible for the oxidation of alkanes in both the animal and plant bacterial systems has been depicted in Fig. 24–17. The mechanism involves an initial hydroxylation

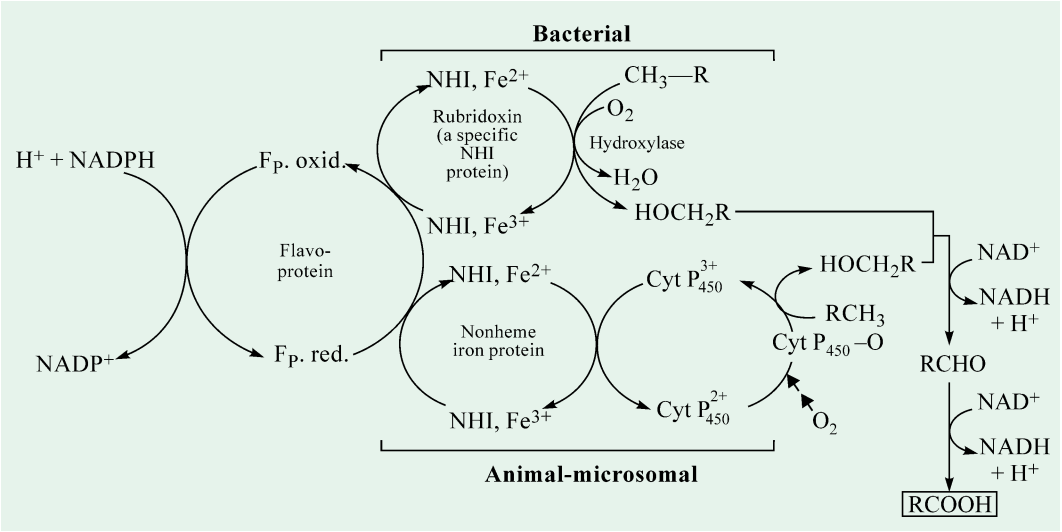


Fig. 24–17. The ω oxidation system responsible for the oxidation of alkanes in bacteria and animal systems

[Fp oxi = flavoprotein oxidized, Fp red = flavoprotein reduced, NHI = Nonheme iron protein]

(*Adapted from Conn EE and StumpfPK, 1976*)

of the terminal methyl group to a primary alcohol. In animals, the cytochrome P_{450} system is the hydroxylase responsible for this alkane hydroxylation ; whereas in bacteria, rubridoxin is the intermediate electron carrier which feeds electrons to ω hydroxylase system. The immediate product, RCH_2OH is oxidized to an aldehyde by an *alcohol dehydrogenase*, which in turn is oxidized to a carboxylic acid by an *aldehyde dehydrogenase* in both systems. Summarily, the —CH_3 group is converted to a —CH_2OH group which subsequently is oxidized to —COOH, thus forming a dicarboxylic acid. Once formed, the dicarboxylic acid may be shortened from either end of the molecule, by the β oxidation sequence, to form acetyl-CoA.

These series of reactions now have assumed an extremely important scavenging role in the bacterial biodegradation of both detergents derived from fatty acids and even more important the large amounts of oil spilled over the ocean surface. The rate of bacterial oxidation of floating oil under aerobic conditions is estimated as high as 0.5g/day per square metre of oil surface. The bacterial oxidation of oils is brought about primarily by ω oxidation mechanism.

KETOGENESIS

General Considerations

The term **ketogenesis** means formation of ketone bodies. The acetyl-CoA, formed in fatty acid oxidation, enters the citric acid cycle if fat and carbohydrate degradation are approximately balanced. The molecular basis of the adage that *fats burn in the flame of carbohydrates* is now evident. The entry of acetyl-CoA into citric acid cycle depends on the availability of oxaloacetate for the formation of citrate. However, if fat breakdown predominates, the acetyl-CoA undergoes a different fate. This is because the concentration of oxaloacetate is lowered if carbohydrate is not available or else poorly utilized. Also, in fasting or in diabetes, the oxaloacetate is utilized in the formation of glucose and is thus unavailable for condensation with acetyl-CoA. Under these conditions, acetyl-CoA is diverted to the formation of acetoacetate (**3-oxobutyrate**, in systematic nomenclature) and **D-3-hydroxybutyrate**. Acetoacetate continually undergoes spontaneous decarboxylation to yield acetone, which is exhaled. The reaction is slow, but if the concentration of acetoacetate becomes high, enough acetone may be formed to make its characteristic odour

detectable in the breath of the individuals. This is the part of the reason that the 3 substances (acetoacetate, D-3-hydroxybutyrate and acetone) were collectively but inaccurately called the "**ketone bodies**" (or "**acetone bodies**") by early investigators even though acetone is the minor part of the total. The term now seems quaint, but it is still in use. And an increase in blood concentrations, of these compounds is called **ketonemia**.

Biosynthesis and Utilization of Ketone Bodies

Biosynthesis. Ketone bodies are formed by a series of unique reactions (Fig. 24–18), primarily in the liver and kidney mitochondria. The enzymes involved in the synthesis of ketone bodies are localized primarily in liver and kidney mitochondria. *Ketone bodies cannot be utilized in the liver since the key utilizing enzyme, β-ketoacyl : CoA transferase (= 3-oxoacid : Coa transferase) is absent in the tissue* but is present in all tissues metabolizing ketone bodies, namely red muscle, cardiac muscle, brain and kidney.

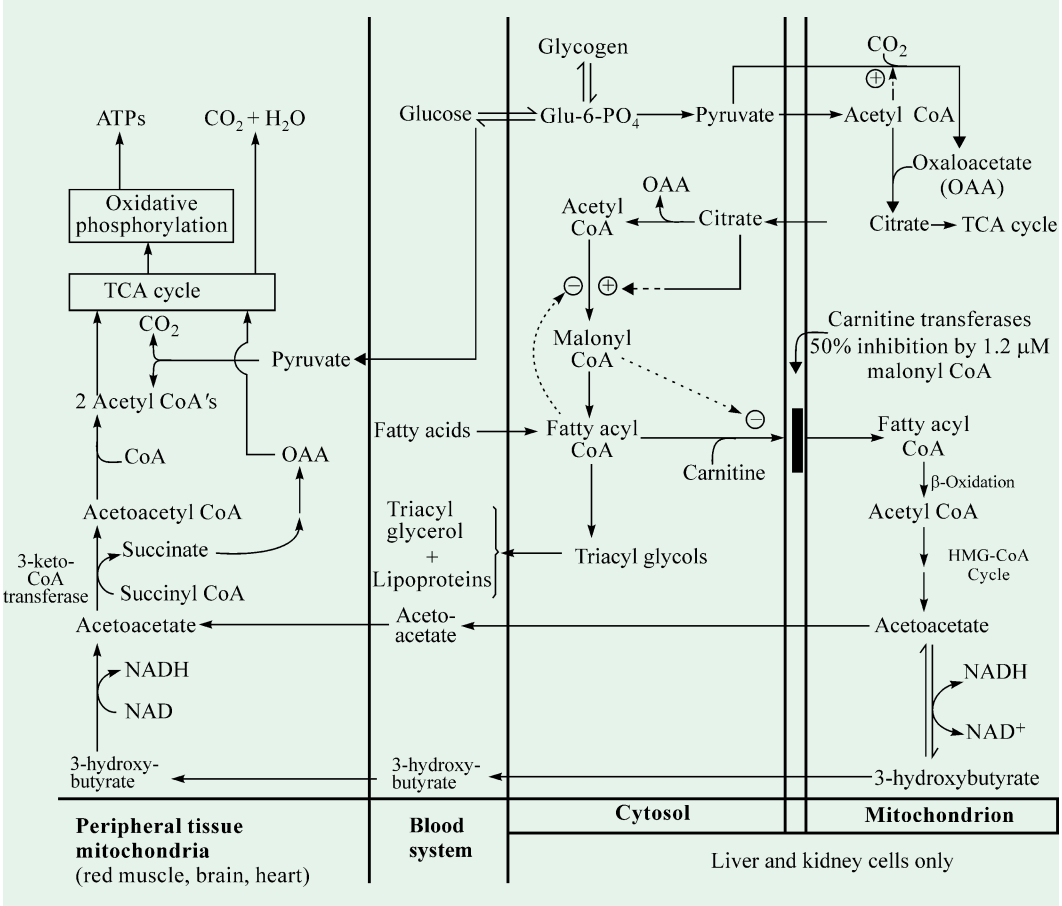


Fig. 24–18. Biosynthesis of ketone bodies and their utilization

Note that the C-6 compound, β-methylglutaryl-CoA (HMG-CoA) is also an intermediate of sterol biosynthesis, but the enzyme that forms HMG-CoA in that pathway is cytosolic. HMG-CoA lyase is present in the mitochondrial matrix but not in the cytosol.

Acetoacetate is produced from acetyl-CoA in the liver and kidneys by a simple three-step process. The first step in the formation of acetoacetate, one of the 3 ketone bodies, is the reversible head-to-tail condensation of 2 moles of acetyl-CoA, to produce a mole of acetoacetyl-CoA enzymatically by *thiolase* (**Step 1**). This steps is simply the reversal of the last step of β

oxidation, *i.e.*, thiolysis. The acetoacetyl-CoA then condenses with acetyl-CoA and water to form β-hydroxy-β-methylglutaryl-CoA, HMG-CoA (**Step 2**). The unfavourable equilibrium in the formation of acetoacetyl-CoA is compensated for by the favourable equilibrium of this reaction, which is due to the hydrolysis of a thioester linkage.

If we think of acetoacetyl-coenzyme A as being analogous to oxaloacetate, we find that the condensation reaction (Step 2) in exactly analogous to the formation of citrate in the first step of the citric acid cycle. However, it is catalyzed by a quite different enzyme, 3-hydroxy-3-methylglutaryl-CoA synthetase. HMG-CoA is ultimately cleaved at a different point to yield acetoacetate and acetyl-CoA in an irreversible reaction (**Step 3**). The sum of these reactions is :

$$2 \text{ Acetyl-CoA} + H_2O \longrightarrow \text{Acetoacetate} + 2 \text{ CoA} + H^+$$

The acetoacetate so produced is reversibly reduced by a mitochondrial enzyme, D-β-*hydroxybutyrate dehydrogenase* to produce D-β-hydroxybutyrate (Fig. 24–18). This enzyme is specific for the D-stereoisomer ; it does not act on L-β-hydroxyacyl-CoAs and is not to be confused with L-β-hydroxyacyl-CoA dehydrogenase, which participates in the β-oxidation pathway. The ratio of hydroxybutyrate to acetoacetate depends on the NADH/NAD^+ ratio inside mitochondria. Accetoacetate is also easily decarboxylated to acetone, either spontaneously or by the action of *acetoacetate decarboxylase*.

Utilization. The acetoacetate and D-β-hydroxybutyrate diffuse from the liver mitochondria into the blood and are transported to peripheral tissues. George Cahill and others have shown that these two acetyl-CoA products are important molecules in energy production. *Acetoacetate and* D-β-*hydroxybutyrate are normal fuels of respiration and are quantitatively important as source of energy*. In fact, heart muscles and renal cortex use acetoacetate in preference to glucose. On the contrary, glucose is the major fuel for the brain in well-nourished persons on a balanced diet. However, brain adapts to utilization of acetoacetate during fasting, starvation and diabetes. In prolonged starvation, 75% of the fuel needs of the brain are met by acetoacetate.

Fatty acids are released by adipose tissue and converted into acetyl units by the liver, which then exports them as acetoacetate. *Acetoacetate can, thus, be regarded as a water-soluble transportable form of acetyl units*.

To sum up, ketone bodies are alternative substrates to glucose, for energy sources in muscle and brain. The precursors of ketone bodies, namely free fatty acids are toxic in high concentrations, have very limited solubility, and readily saturate the carrying capacity of the plasma membrane. On the other hand, the ketone bodies are low in toxicity, and tolerated at high concentrations, are very soluble, diffuse rapidly through membranes, and are rapidly metabolized to CO_2 and H_2O.

The importance of this pathway is indicated by the fact that the normal human liver is potentially capable of making the equivalent of half its weight as acetoacetate each day !

In the extrahepatic tissues, D-β-hydroxybutyrate is oxidized to acetoacetate by an enzyme, D-β-*hydroxybutyrate dehydrogenase*. Acetoacetate is activated to form its coenzyme A ester by transfer of CoA from succinyl-CoA, an intermediate of the citric acid cycle, in a reaction catalayzed by b-ketoacyl-CoA transferase. The acetoacetyl-CoA is then cleaved by thiolase to yield 2 moles of acetyl-CoA, which enter the citric acid cycle.

Ketogenic and Antiketogenic Substances

The *ketogenic substances* are, of course, all the fatty acids. In addition, at least 3 amino acids belong to this category : leucine, phenylalanine and tyrosine. *Antiketogenic substances*, in the sense of preventing the accumulation of ketone bodies, are the carbohydrates, the glycerol fraction of fat, and the following amino acids : glycine, alanine, valine, serine, threonine, cysteine, methionine, aspartic acid, glutamic acid, arginine, histidine, proline and ornithine. These are antiketogenic because their non-nitrogenous residues are convertible to glucose.

Regulation of Ketogenesis

Ketosis arises as a result of deficiency in available carbohydrate. This leads to the enhanced rate of ketogenesis. There are 3 crucial steps in the pathway of metabolism of free fatty acids (FFAs) that determine the magnitude of ketogenesis (Fig. 24–19). These are :

1. It causes an imbalance between esterification and lipolysis in adipose tissue, with consequent of free fatty acids into the circulation. FFAs are the principal substrates for ketone body formation in the liver and therefore all factors, metabolic or endocrine, affecting the release of free fatty acids from adipose tissues, influence ketogenesis.
2. Upon entry of free fatty acids into the liver, the balance between esterification and oxidation of FFAs is influenced by the hormonal state of the liver and possibly by the availability of glycerol-3-phosphate or by the redox state of the tissue.

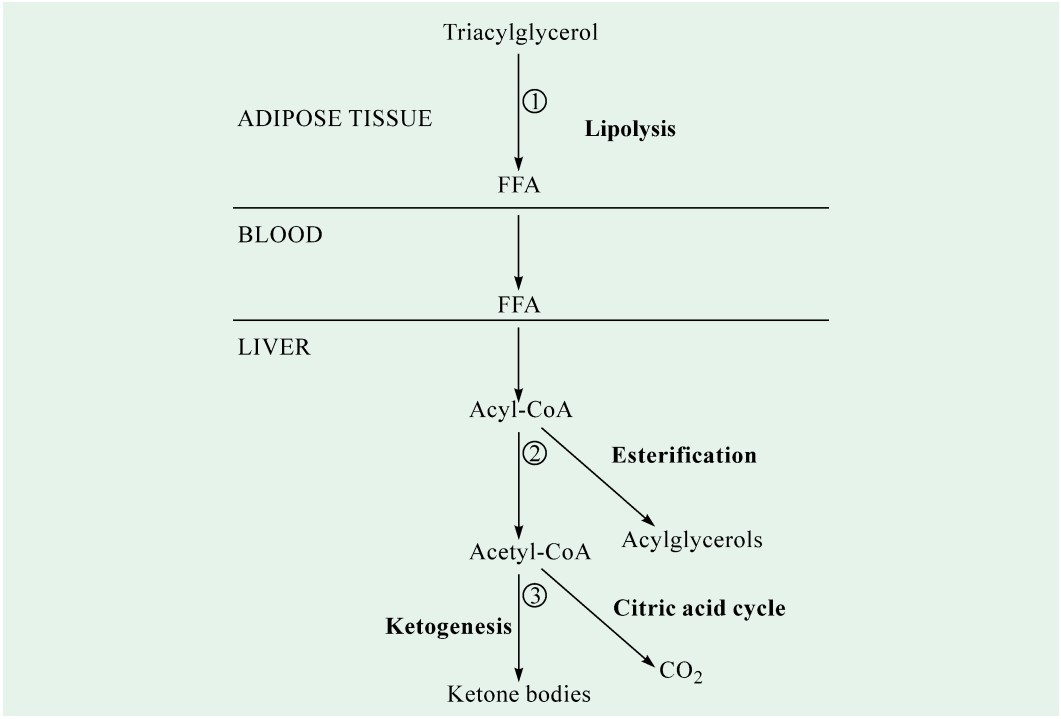


Fig. 24–19. Regulation of ketogenesis

The 3 crucial steps in the pathway of free fatty acids (FFAs) that determine the rate of ketogenesis are numbered as ①, ②, and ③.

(*After Harper, Rodwell and Mayes, 1977*)

3. As the quantity of fatty acids presented for oxidation increases, more form ketone bodies and less form CO_2, regulated in such a manner that the total energy production remains constant.

Under circumstances of limited utilization of carbohydrates and/or substantial mobilization of fatty acids to the liver, there is a markedly diminished rate of operation of two of the three pathways for metabolizing acetyl-CoA, *viz*., the citric acid cycle and fatty acid synthesis. The result is a channeling of acetyl-CoA into β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). This results in increased formation of acetoacetate, D-β-hydroxybutyrate and acetone, whereby elevating the concentration of the total ketone bodies in the blood and urine above normal (Table 24–1). Higher than normal quantities of the ketone bodies present in the blood or urine constitute ketonemia

(= hyperketonemia) and ketonuria, respectively. D-β-hydroxybutyrate is quantitatively the predominant ketone body present in the blood and urine in ketosis. Whenever a marked degree of ketonemia and ketonuria exists, the odour of acetone is likely to be detected in the exhaled air. The triad of ketonemia, ketonuria and acetone odour of the breath is collectively termed **ketosis.**

Table 24–1. Accumulation of ketone bodies in untreated diabetics with severe ketosis

State of individual	*Blood concentration* (mg/100 mL)*	*Urinary excretion (mg/24 hr)*
Normal	<3	≤ 125
Extreme ketosis (untreated diabetic)	90	5,000

*as acetone equivalents

Since H^+ is produced along with oxybutyrates, ketosis is frequently accompanied by acidosis. **Acidosis** is the lowering of blood pH due to the rise in blood levels of acetoacetate and D-β-hydroxybutyrate. Ketosis and acidosis taken together are termed as **ketoacidosis**. The ketoacidosis is characterized by the continual excretion in quantity of the ketone bodies which entails some loss of buffer cation, thus progressively depleting the alkali reserve. Ketoacidosis is of frequent occurrence in the untreated juvenile diabetic because of failure to secrete sufficient insulin and may be fatal. In late-onset diabetes after age 40, severe ketoacidosis is less frequent.

FATTY ACID OXIDATION IN PEROXISOMES

Although the major site of fatty acid oxidation in animal cells is the mitochondrial matrix, other compartment in certain cells also contain enzymes which are capable of oxidizing fatty acids to acetyl-CoA, by a pathway similar to, but not identical with, that in mitochondria. **Peroxisomes** are membrane-bound cellular compartments in animals and plants. The fatty acids are oxidized in peroxisomes to produce hydrogen peroxide which is then destroyed enzymatically. As in the oxidation of fatty acids in mitochondria, the intermediates are coenzyme A derivatives. The whole process consists of 4 steps (Fig. 24–20) :

(*a*) dehydrogenation of long-chain acyl-coenzyme A,

(*b*) addition of water to the resulting double bond,

(*c*) oxidation of the β-hydroxyacyl-CoA to a ketone, and

(*d*) thiolytic cleavage by coenzyme A.

The fatty acid oxidation in **glyoxysomes** occurs by the peroxisomal pathway.

The peroxisomal pathway differs from the mitochondrial pathway in 3 respects:

1. In peroxisomal pathway, the flavoprotein dehydrogenase, that introduces the double bond, passes electrons directly to O_2, producing H_2O_2. This strong and potentially damaging oxidant is immediately cleaved to H_2O and O_2 by *catalase*. Whereas in mitochondrial pathway, the electrons removed in first oxidative step pass through the respiratory chain to O_2, forming H_2O. This process is accompanied by ATP synthesis. In peroxisomes, the energy released in the first oxidative step of fatty acid breakdown is dissipated as heat.

2. The NADH formed in β oxidation cannot be reoxidized, and the peroxisomes must export reducing equivalent to the cytosol (These equivalents are eventually passed on to mitochondria).

3. In mitochondria, the acetyl-CoA is further oxidized via the citric acid cycle. Acetyl-CoA produced by peroxisomes (and also glyoxysomes) is exported. The acetate from glyoxysomes serves as a biosynthetic precursor of polysaccharides, amino acids, nucleotides and some metabolic intermediates.

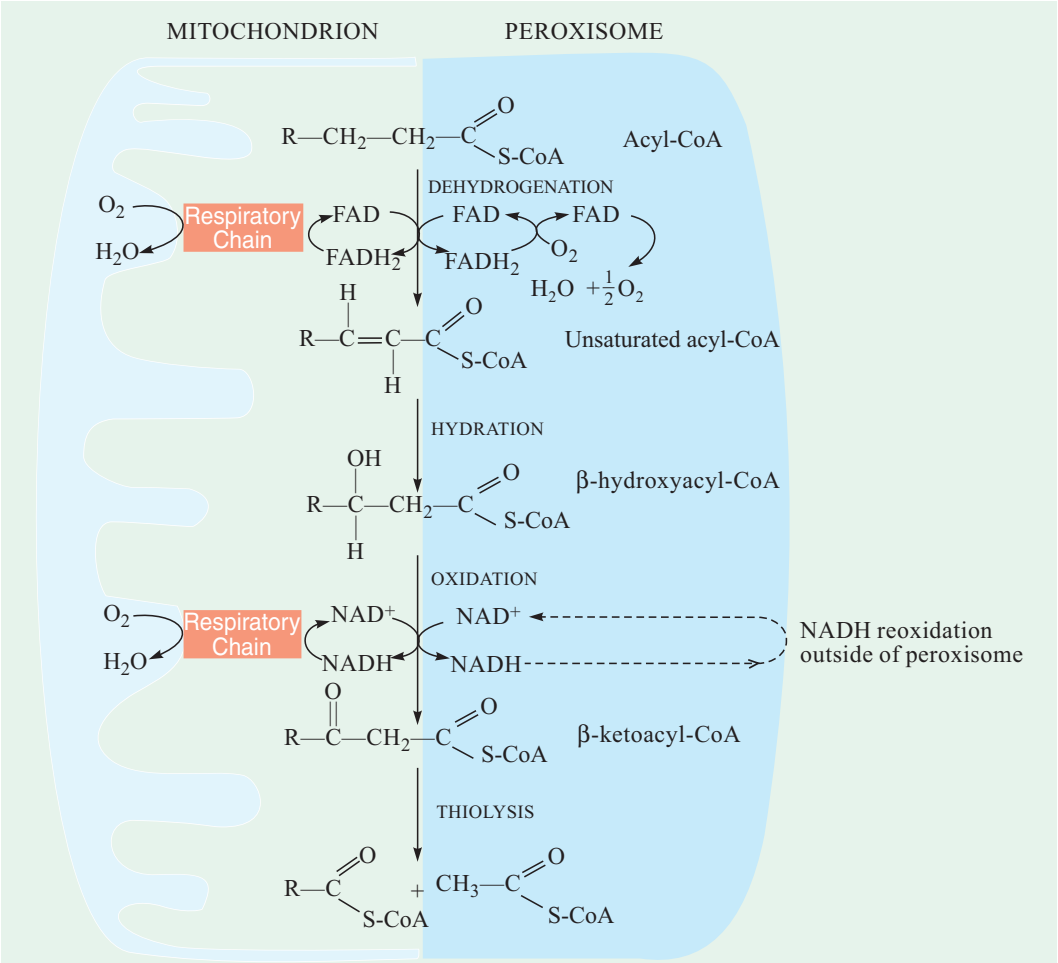


Fig. 24–20. Comparison of β oxidation of fatty acids as it occurs in animal mitochondria and in animal and plant peroxisomes

METABOLIC WATER

Another important biological feature of fatty acid oxidation (and of aerobic respiration, in general) is the production of metabolic water. For example, a mole of palmitic acid upon oxidation

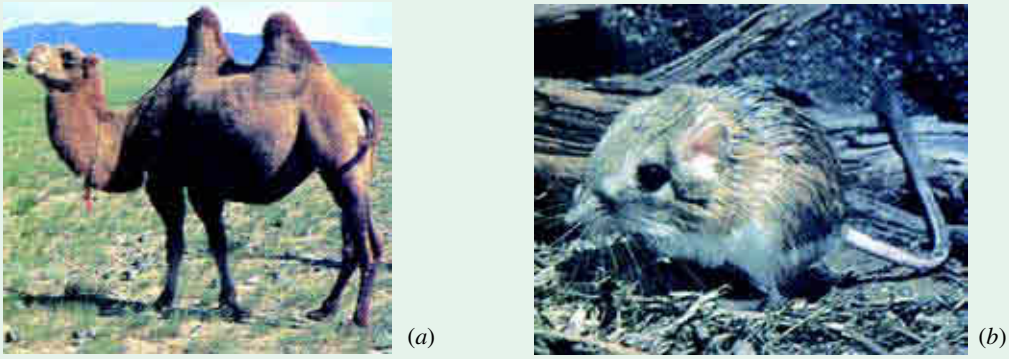


Fig. 24–21. Adaptation of animals to arid environment
(a) Camel (b) Kangaroo rat

produces 16 moles of H_2O. This metabolic production of water is of significant importance to many organisms (Fig. 24–21). In the case of a **camel**, for example, the lipids stored in its humps serve both as a source of energy and as a source of the water needed to help sustain the animal for extended periods of time in the desert. The **kangaroo rat** is an even more striking example of adaptation to an arid environment. This animal has been observed to live indefinitely without having to drink water. It lives on a diet of seeds, which are rich in lipids but contain little water. The metabolic water that the kangaroo rat produces is adequate for all its water needs. This metabolic response to arid conditions is usually accompanied by a reduced output of urine.

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