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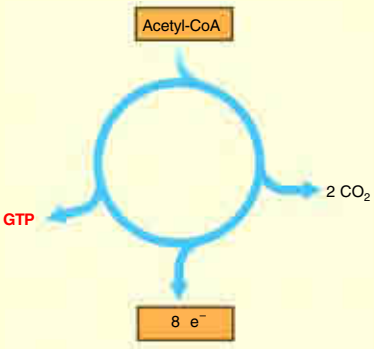


Roundabouts, or traffic circles, function as hubs to facilitate traffic flow
The citric acid cycle is the biochemical hub of the cell, oxidizing carbon fuels, usually in the form of acetyl-CoA, as well as serving as a source of precursors for biosynthesis.
(Courtesy : (Upper) Chris Warren)

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.

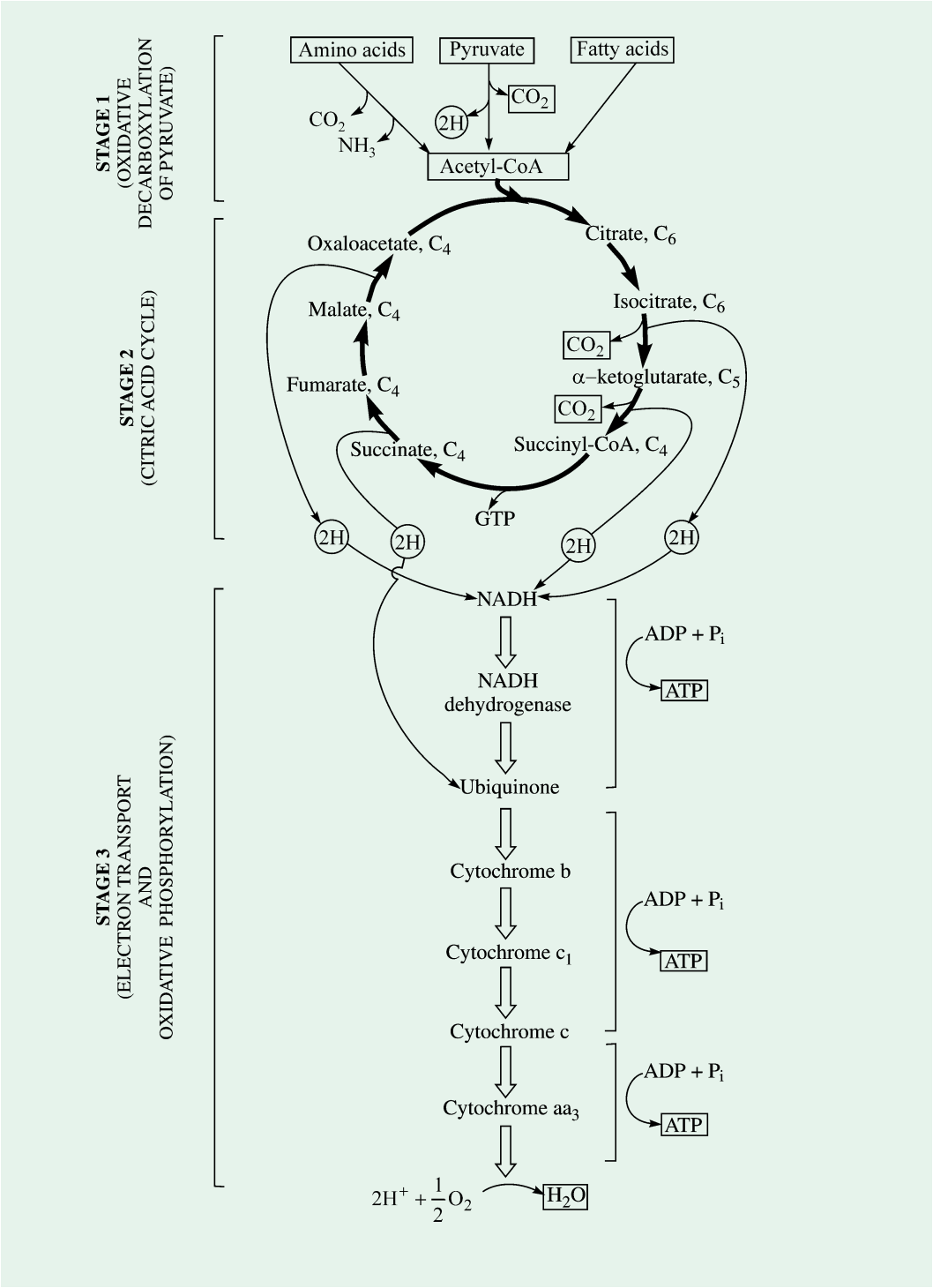


Fig. 22-1. Three stages in cell respiration

(*Adapted from Lehninger AL, 1984*)

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

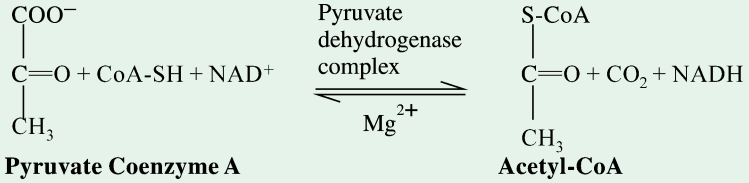
In this stage, the acetyl groups so obtained are fed into the citric acid cycle (= Krebs' cycle) which degrades them to yield energy-rich hydrogen atoms and to release CO_2, the final oxidation pruduct of organic fuels. It is, thus, the final common pathway for oxidation of fuel molecules. The cycle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

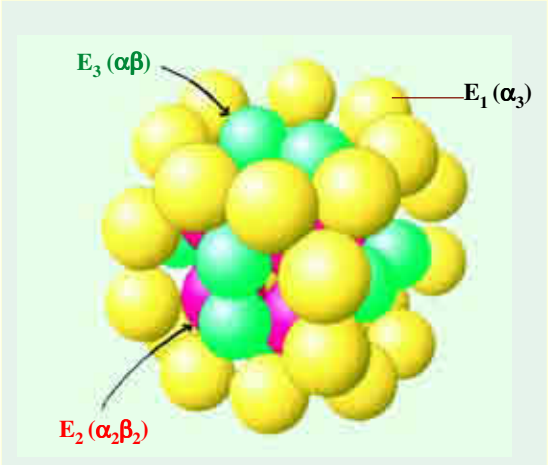


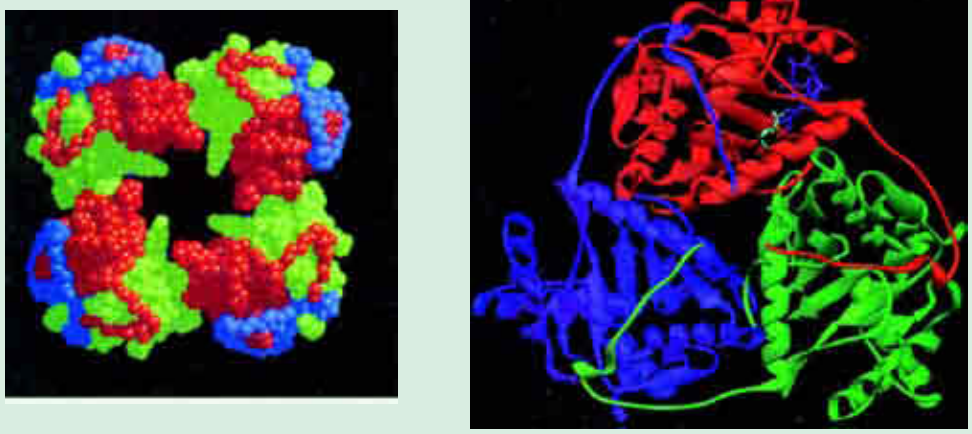
Fig. 22-2. Space-filling model of the pyruvate dehydrogenase complex

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)

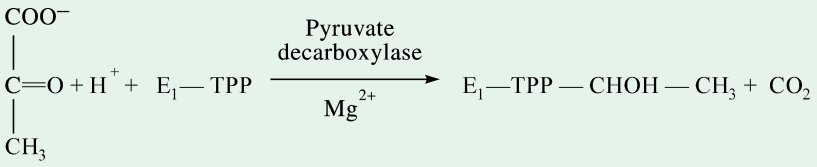
and dihydrolipoyl dehydrogenase contains bound FAD. The lipoyllysyl groups of the 'core' enzyme serve as `swinging or flexible arms' that can transfer hydrogen atoms and acetyl groups from one enzyme molecule to another in the multienzyme complex. All these enzymes and coenzymes are organized into a 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 :

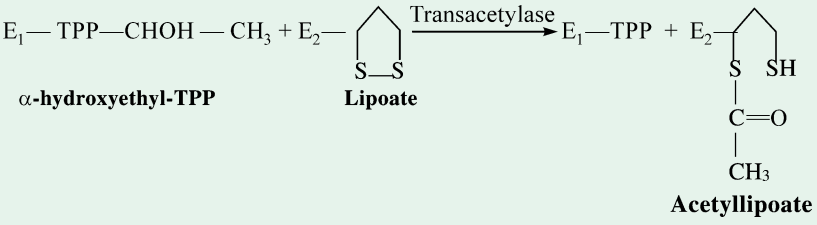
Stage 1 :

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.



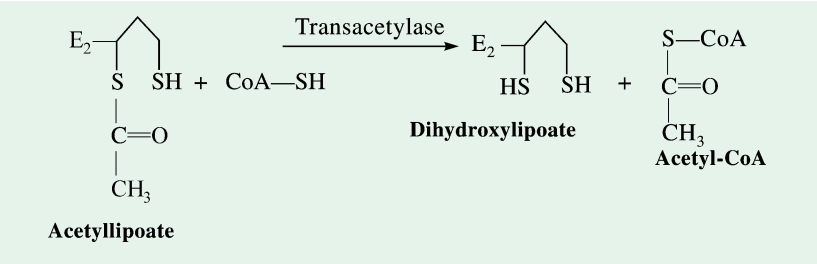
Stage 2 :

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.



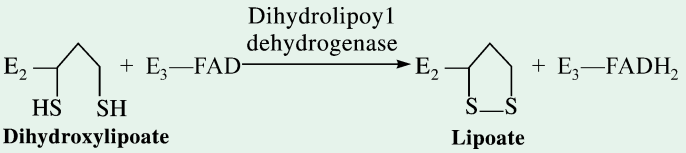
Stage 3 :

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.



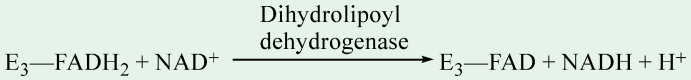
Stage 4 :

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



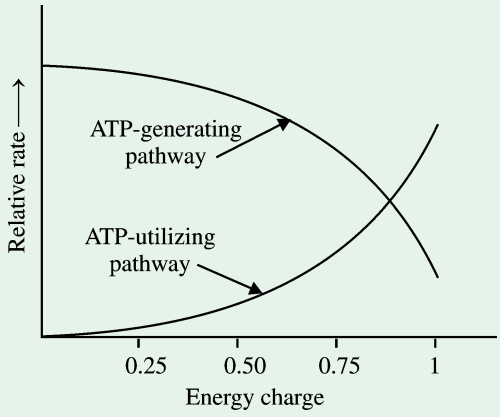
Regulation of Oxidative Decarboxylation of Pyruvate

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:

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

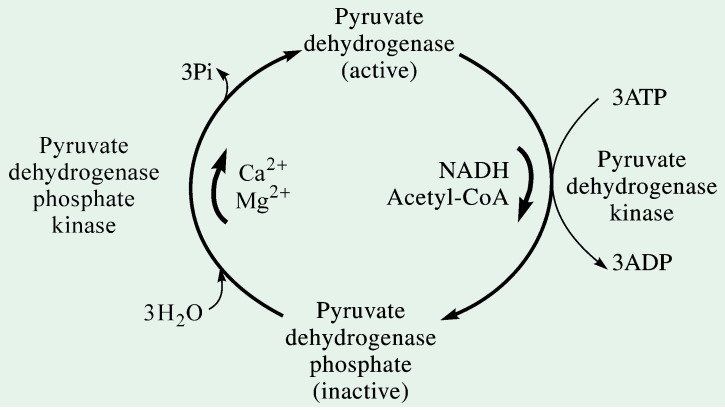
The phosphorylation potential, contrary to the energy charge, depends on the concentration of Pi and is directly related to the free energy-storage available from ATP.

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.

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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).

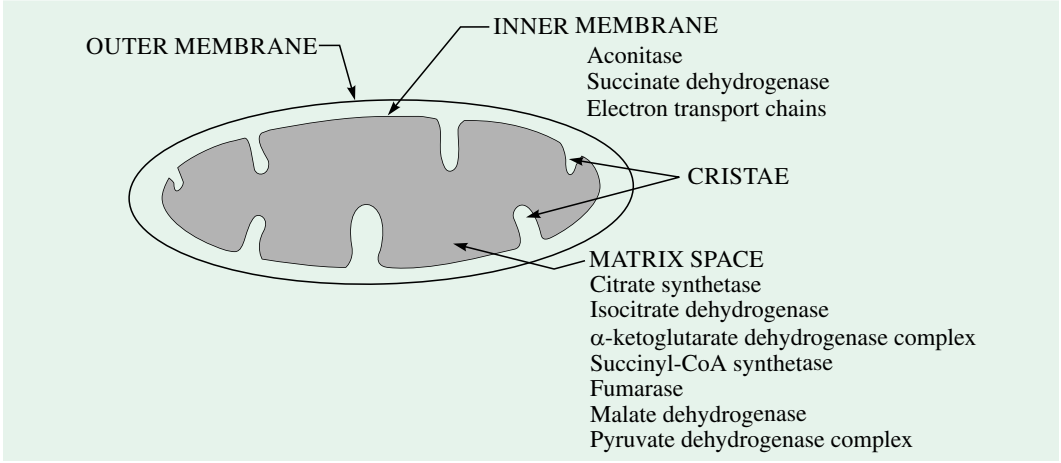


Fig. 22–5. Location of citric acid cycle enzymes in mitochondria

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.

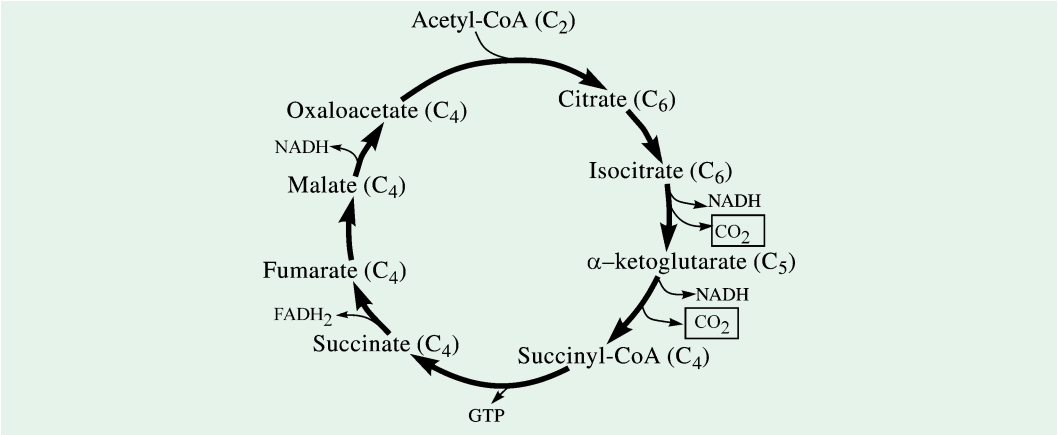


Fig. 22-6. An outline of the citric acid cycle

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**

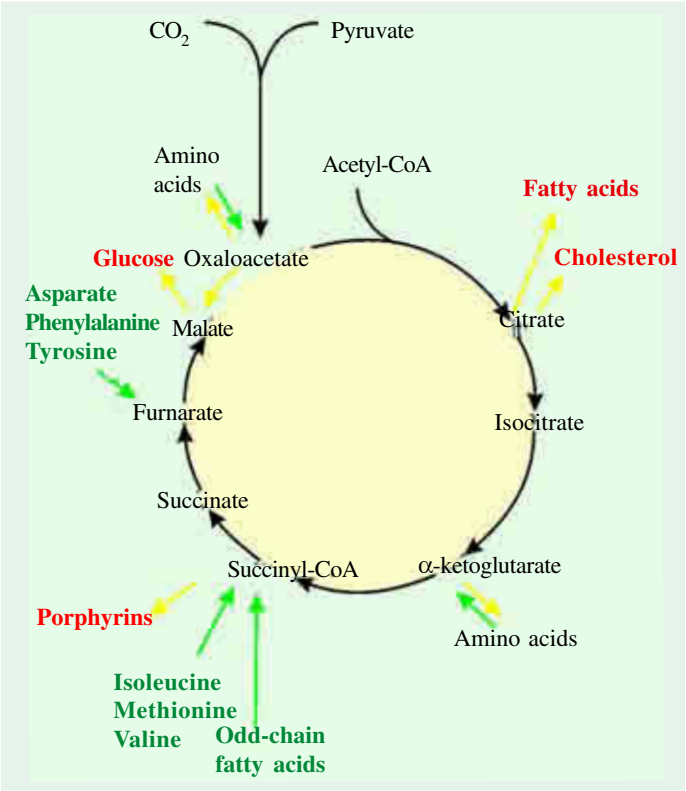
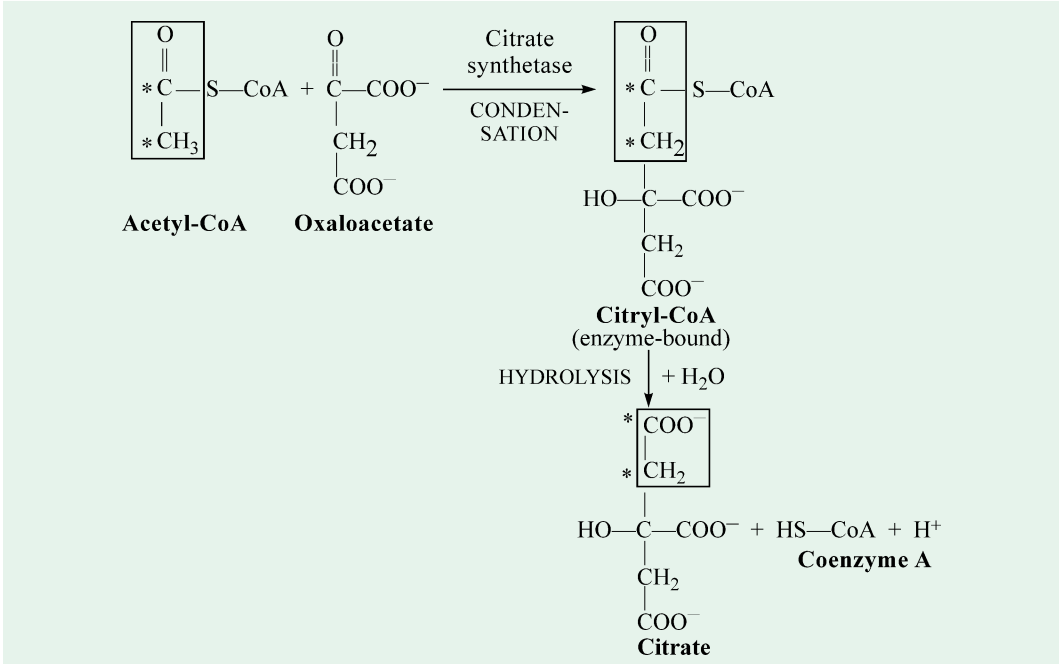


Fig. 22–7. A diagram of the citric acid cycle, indicating the positions at which intermediates are drawn off for use in anabolic pathways *(red arrows)* **and the points where anaplerotic reactions replenish depleted cycle intermediates** *(green arrows)***.**

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

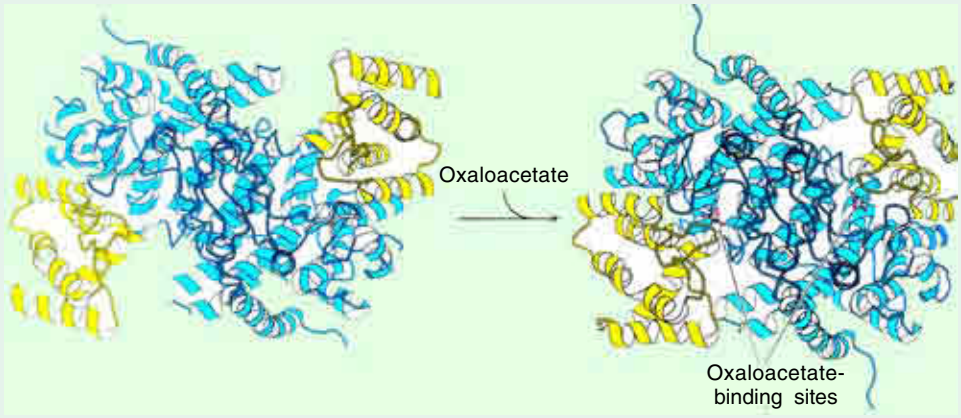


Fig. 22–8. Ribbon model of the conformational changes in citrate synthase on binding oxaloacetate The small domain of each subunit of the homodimer is shown in *yellow*, the large domain is shown in *blue* (*left*) Open form of enzyme alone. (*Right*) Closed form of the liganded enzyme.

(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.

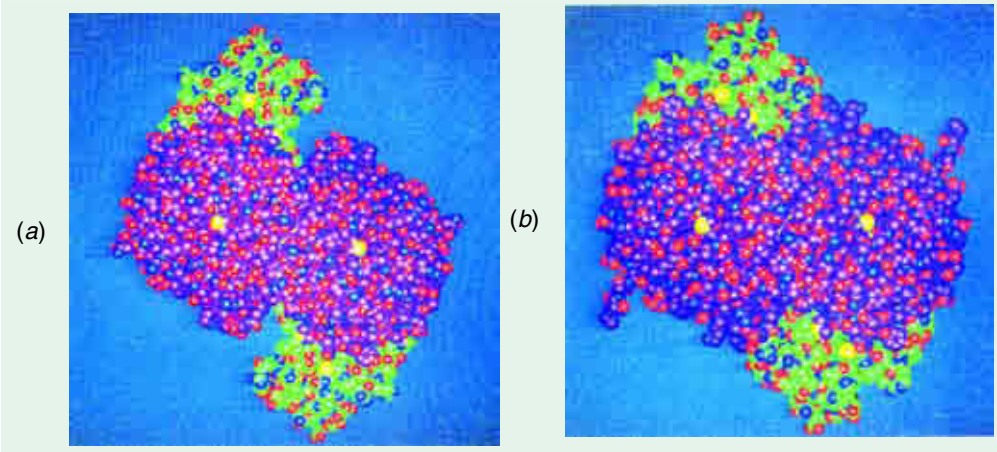
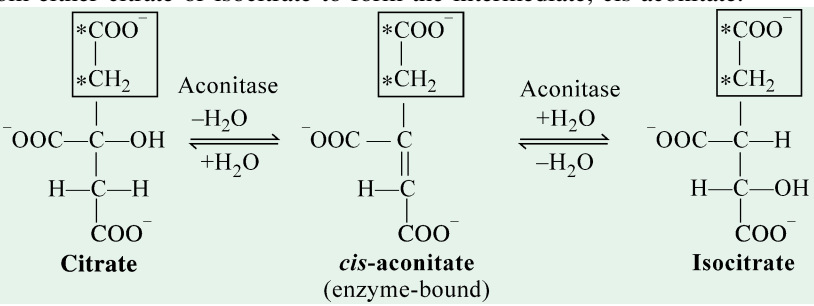


Fig. 22–9. A space-filling drawing of citrate synthase in (*a*) the open conformation and (*b*) the closed, substrate-binding conformation

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.



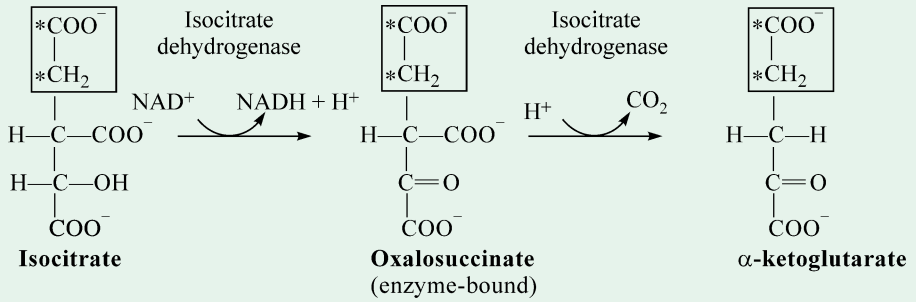
It is possible that cis-aconitate may not be an obligatory intermediate between citrate and isocitrate but may, in fact, be a side branch from the main pathway.

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.

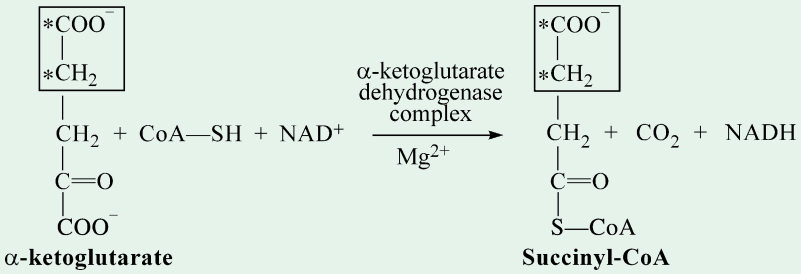
There are 2 types of **isocitrate dehydrogenase**, one requiring NAD^+ as electron acceptor (*NAD^+-specific*) and the other requiring *$NADP^+$* ($NADP^+$-*specific*). Both the types appear to participate in the citric acid cycle, but the NAD^+-specific isocitrate dehydrogenase is predominant. The NAD^+-specific 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.

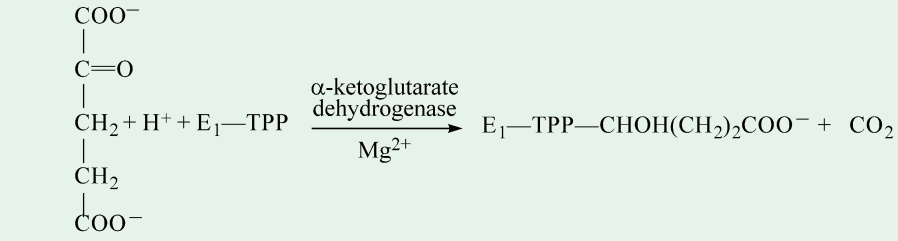
The **α-ketoglutarate dehydrogenase complex** from pig heart (MW = 33,00,000) is much smaller than the PDC from the same source (MW = 1,00,00,000). The pig heart α-KDC is a large multienzyme cluster consisting of 3 enzyme components, *viz.*, α-ketoglutarate dehydrogenase or α-ketoglutarate decarboxylase (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.

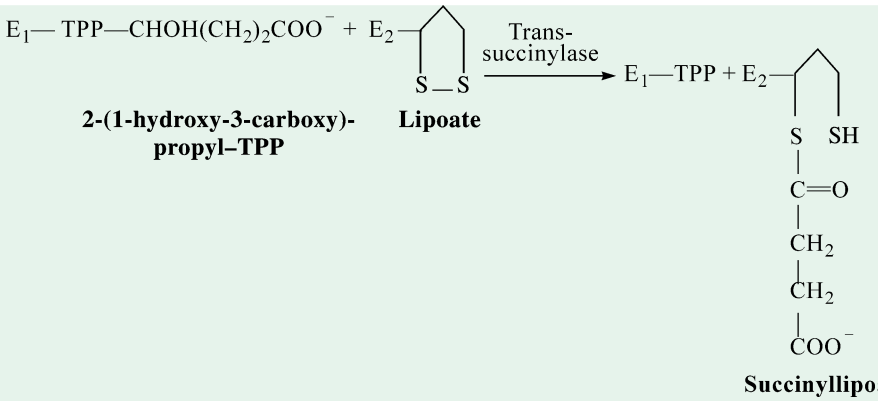
There are 5 successive stages in the conversion of a-ketoglutarate to succinyl-CoA. These are as follows :

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.



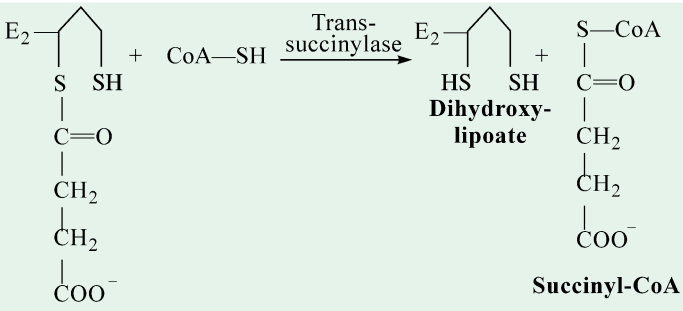
α-ketoglutarate **2-(1-hydroxy-3-carboxy)-propy1–TPP**

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.



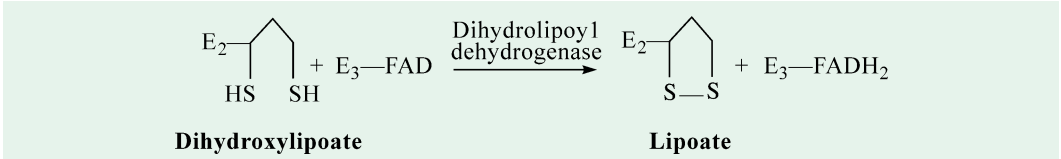
2-(1-hydroxy-3-carboxy)-propyl–TPP **Lipoate** **Succinyllipoate**

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.

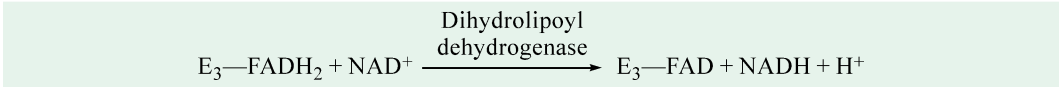


Succinyllipoate **Dihydroxy-lipoate** **Succinyl-CoA**

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.

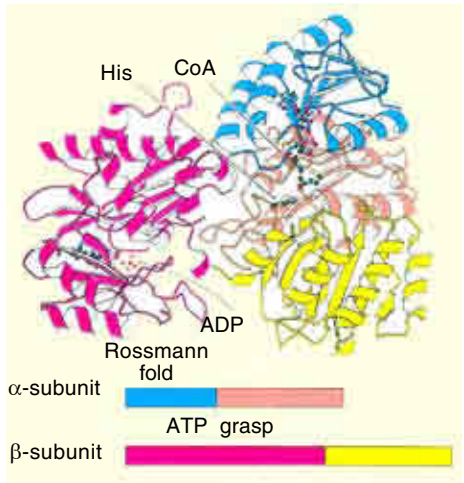
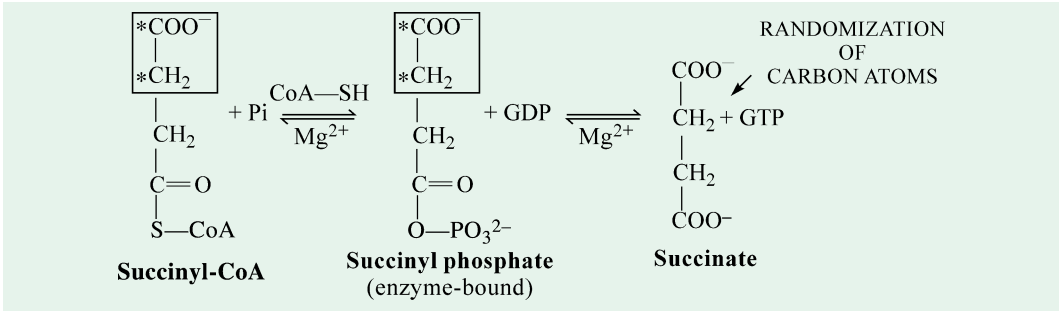


Fig. 22–10. Ribbon model of the structure of succinyl CoA synthetase
The enzyme is composed of two subunits. The α subunit contains a Rossmann fold that binds the ADP component of CoA, and the β subunit contains a nucleotide-activating region called the ATP-grasp domain. The ATP-grasp domain is shown 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).

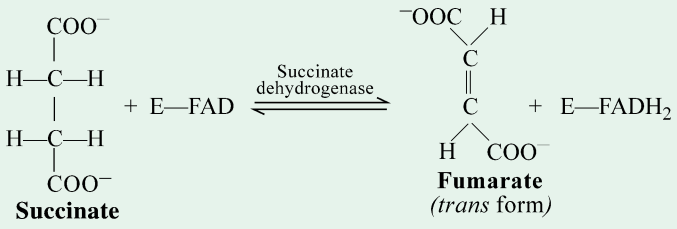
$$GTP + ADP \underset{Mg^{2+}}{\overset{\text{Nucleoside diphosphokinase}}{\rightleftharpoons}} GDP + ATP$$

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

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.



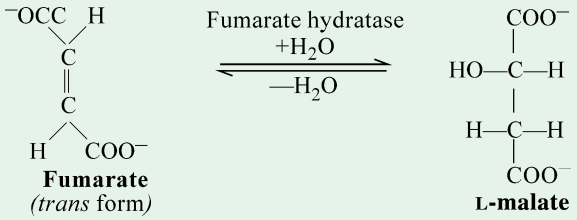
Succinate dehydrogenase from beef heart (MW = 97,000) consists of 2 subunits of unequal size. There is no heme in this enzyme. Rather, the iron atoms are bonded to the inorganic sulfide, resulting in the formation of iron-sulfur clusters or centres of varied molecular arrangement. The large subunit (MW = 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*

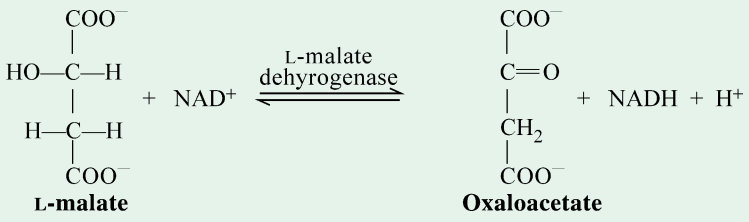
Fumarate is hydrated to form L-malate in the presence of *fumarate hydratase* (formerly known as *fumarase*). This is a freely reversible reaction ($\Delta G^{\circ\prime} \approx 0.0$ kcal/mol) and involves hydration in malate formation and dehydration in fumarate formation. *Fumarate hydratase is highly 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

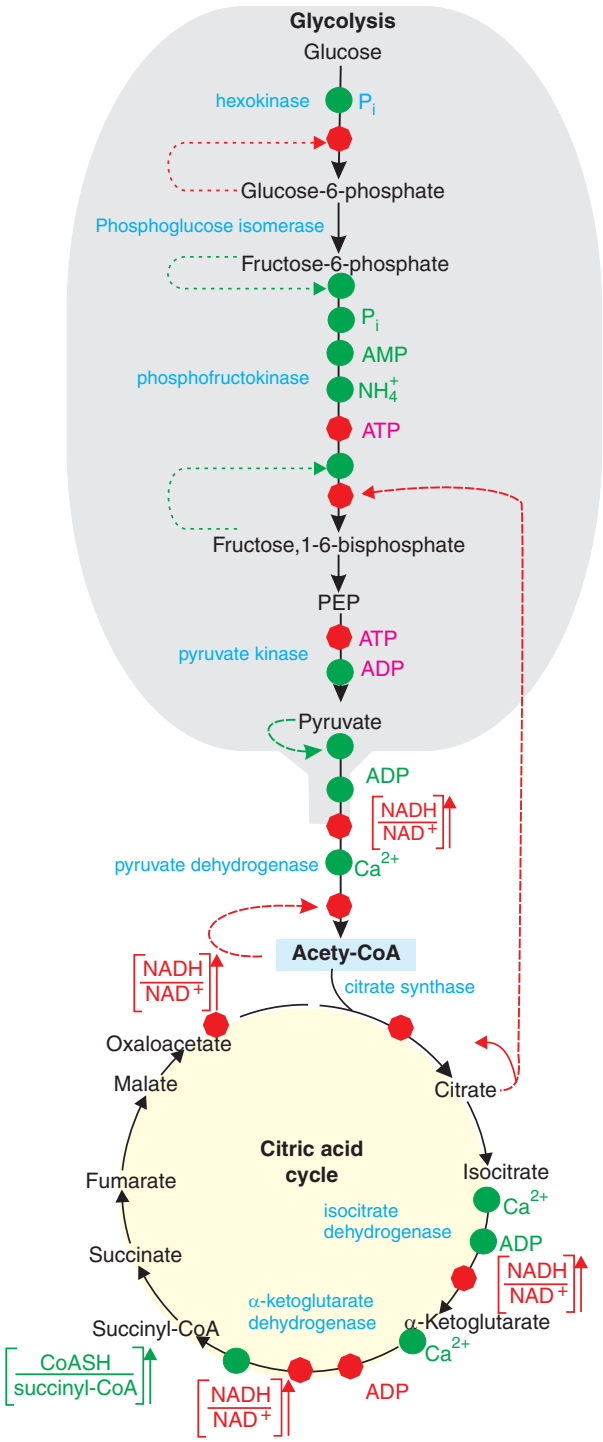


Fig. 22–11. A schematic of the coordinated control of glycolysis and the citric acid cycle by ATP, ADP, AMP, P_i, Ca^{2+}, and the [NADH]/[NAD^+] ratio

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.

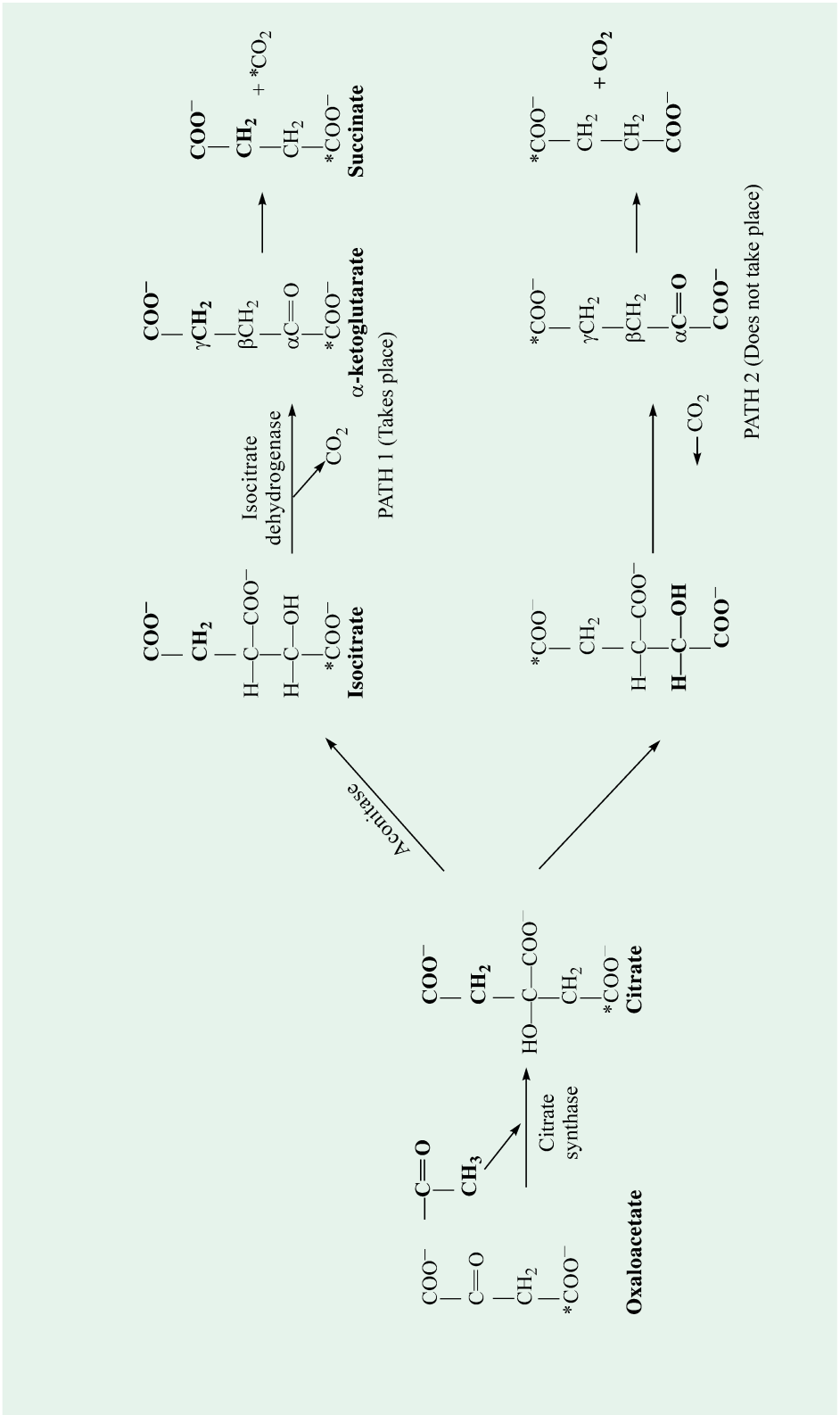


Fig. 22–12. Incorporation of isotopic carboxylate carbon (furthest from the keto group) of oxaloacetate into a-ketoglutarate

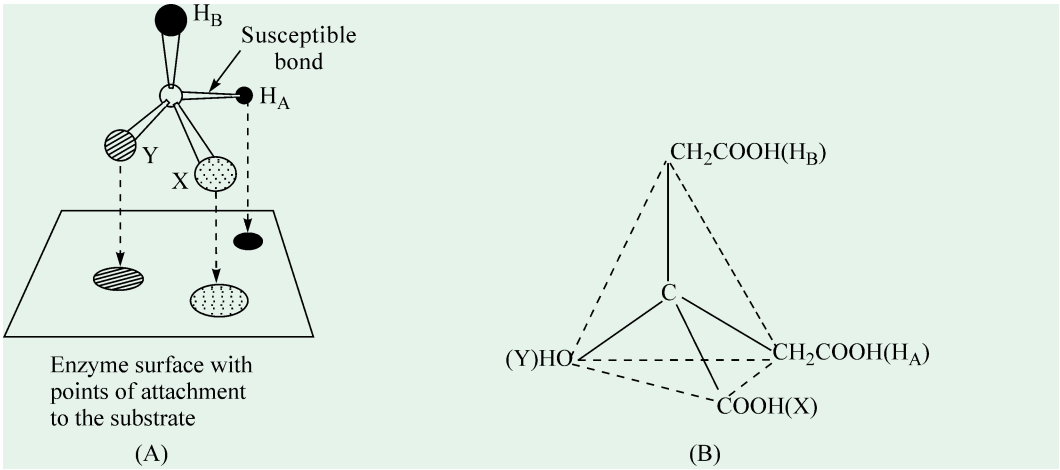


Fig. 22–13. Asymmetry of citrate and the action of aconitase
(A) Ogston's 3-point attachment hypothesis
(B) Structure of citrate

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

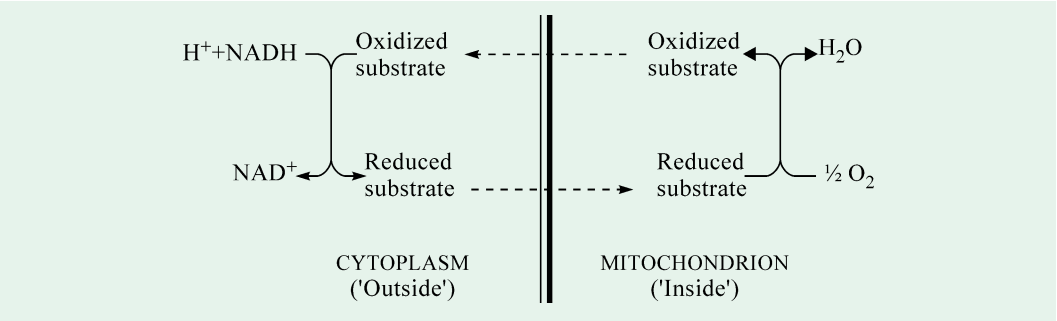
Respiration rate depends, not only on the nature and concentration of the substrates to be oxidized, but also on the coupling of respiration to phosphorylation. Intact mitochondria are usually 'tightly' coupled, so that their rate of respiration is actually controlled by the ratio [ADP]/[ATP]. When this 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.*

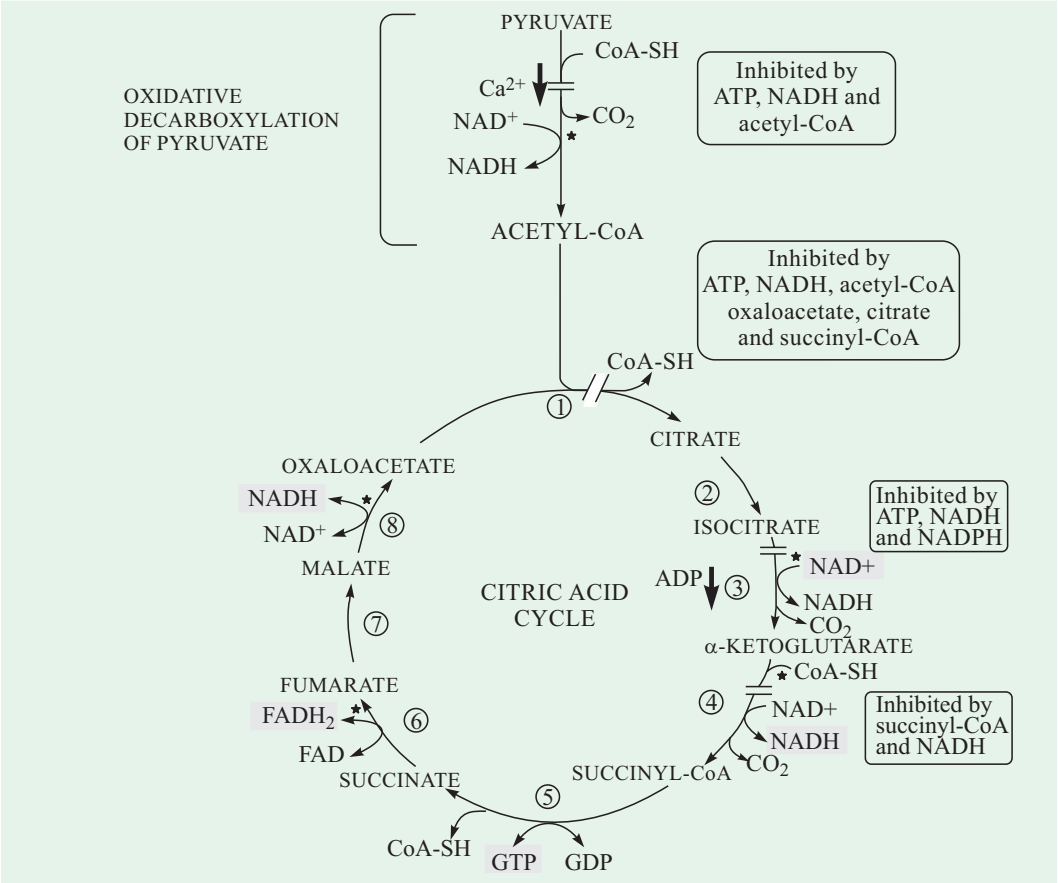


Fig. 22–14. Control of the oxidative decarboxylation of pyruvate and the citric acid cycle

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.

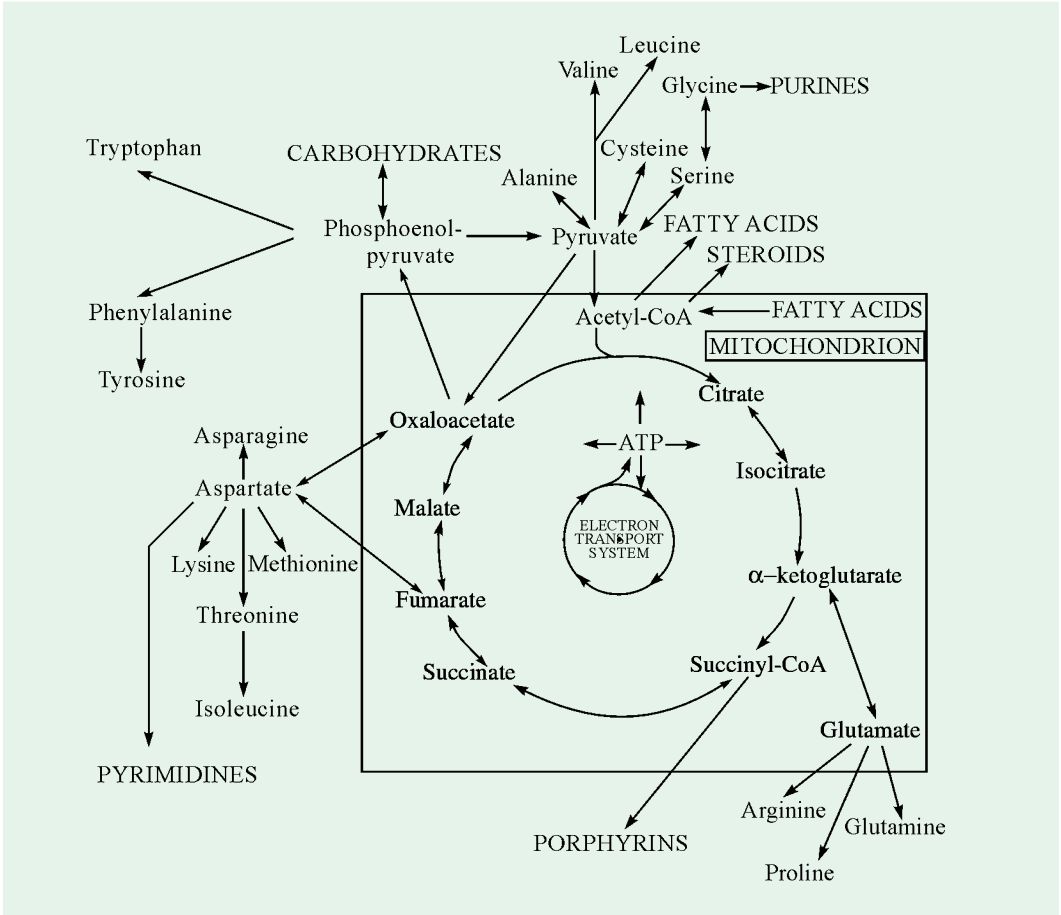


Fig. 22–15. Biosynthetic and anaplerotic functions of the citric acid cycle

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.

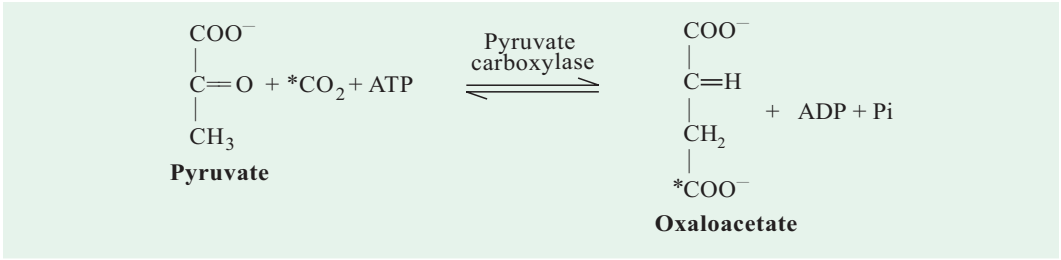
4. Purines and pyrimidines. Purines and pyrimidines are important constituents of the coenzymes and nucleic acids. Aspartate provides the carbon skeleton of pyrimidines whereas glycine from pyruvate contributes the carbon skeleton for purines.

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 :

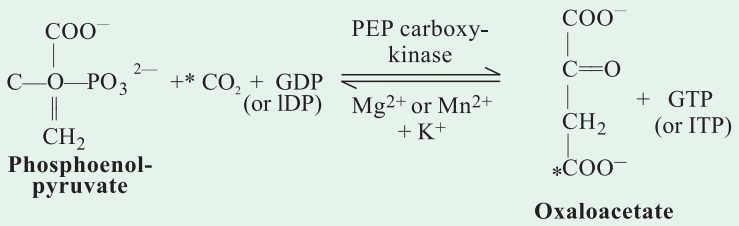
(*a*) **First step :** Free CO_2, the precursor of new carboxyl group of oxaloacetate, is first, 'energized' through its covalent union with a ring N atom of biotin, forming 1′-N-carboxybiotinyl enzyme. The energy needed is provided by ATP. The rate of this reaction is accelerated by acetyl-CoA which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

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

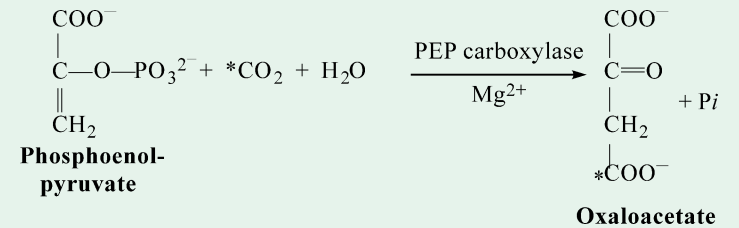
2. Phosphoenolpyruvate carboxykinase reaction. This is another anaplerotic reaction that feeds the Krebs cycle and occurs in animal tissues, *esp*., heart and muscles. This reversible reaction brings about carboxylation of phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) with the concomitant use 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**

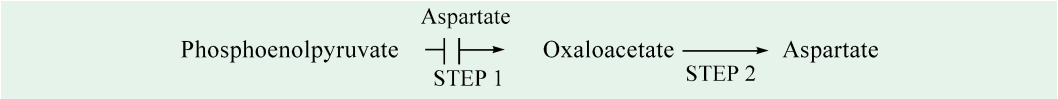
PEP carboxykinase (MW = 75,000) is found primarily in cytosol and to less extent in mitochondria. It differs in mechanism from that of pyruvate carboxylase as it does not involve biotin and also CO_2 is not 'activated'.

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).*

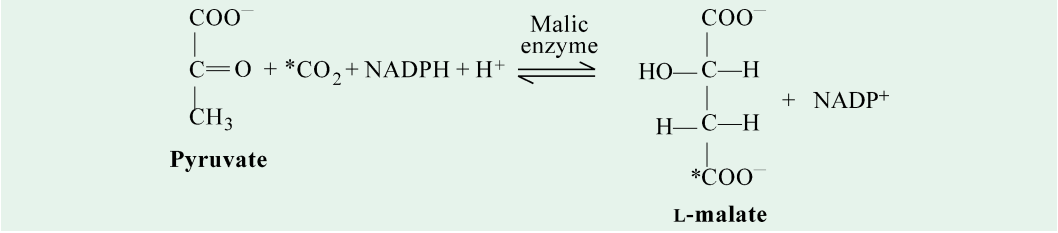


Phosphoenol-pyruvate — **Oxaloacetate**

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 :

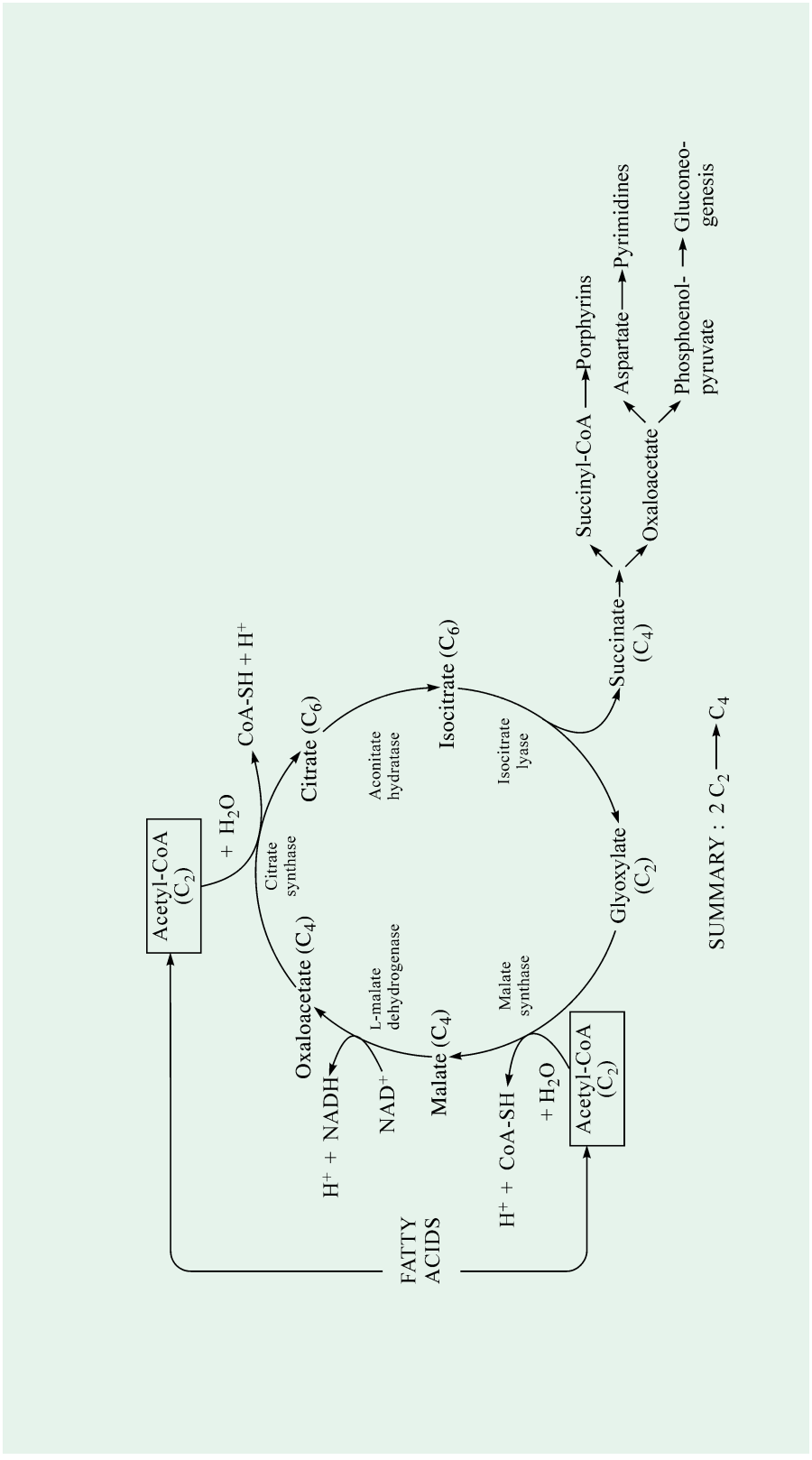


Fig. 22–16. The glyoxylate cycle

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*

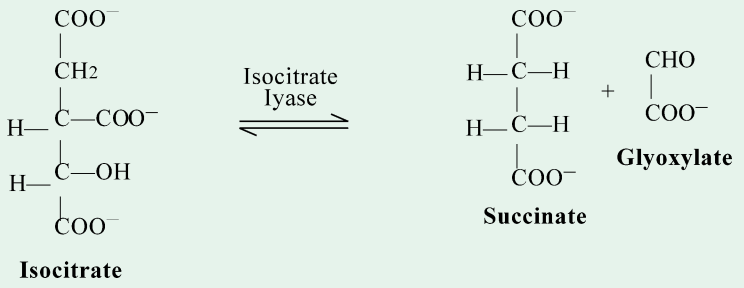
This reaction is catalyzed by the enzyme, *citrate synthase* (For details, see page 394).

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).

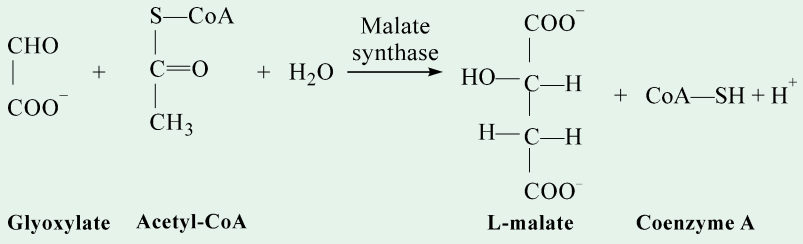
Step 3 : *Cleavage of isocitrate into succinate and glyoxylate*

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.

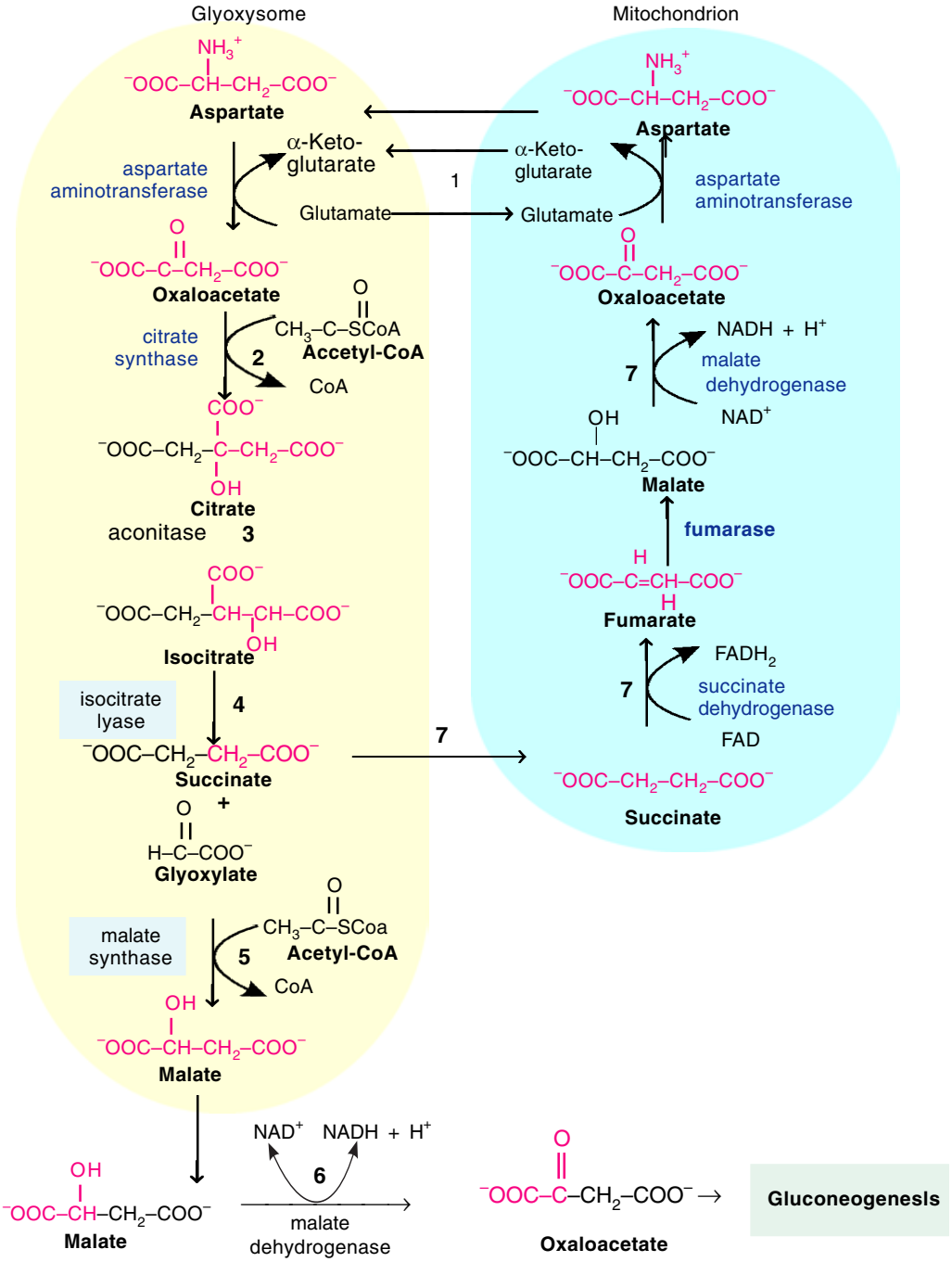


Fig. 22–17. Participation of both mitochondrial and glyoxysomal enzymes in the glyoxylate pathway
Isocitrate lyase and malate synthase, enzymes unique to plant glyoxysomes, are boxed. The pathway results in the net conversion of two acetyl-CoA to oxaloacetate. (1) Mitochondrial oxaloacetate is converted 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*