EVOLUTION OF THE BIOSYNTHESIS OF THE BRANCHED-CHAIN AMINO ACIDS

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Abstract. The origin of the biosynthetic pathways for the branched-chain amino acids cannot be understood in terms of the backwards development of the present acetolactate pathway because it contains unstable intermediates. We propose that the first biosynthesis of the branched-chain amino acids was by the reductive carboxylation of short branched chain fatty acids giving keto acids which were then transaminated. Similar reaction sequences mediated by nonspecific enzymes would produce serine and threonine from the abundant prebiotic compounds glycolic and lactic acids. The aromatic amino acids may also have first been synthesized in this way, e.g. tryptophan from indole acetic acid. The next step would have been the biosynthesis of leucine from α-ketoisovaleric acid. The acetolactate pathway developed subsequently. The first version of the Krebs cycle, which was used for amino acid biosynthesis, would have been assembled by making use of the reductive carboxylation and leucine biosynthesis enzymes, and completed with the development of a single new enzyme, succinate dehydrogenase. This evolutionary scheme suggests that there may be limitations to inferring the origins of metabolism by a simple back extrapolation of current pathways.

1. Introduction

In the first discussion of the origin of biosynthetic pathways, Horowitz (1945) proposed that biosynthetic pathways arose by backwards development rather than forwards. The basis of the proposed process was the utilization of a prebiotic soup that contained all of the biosynthetic intermediates which constitute the resultant pathway. When a required compound (eg threonine) became exhausted from the environment, the preceding intermediate (homoserine) would have been converted to threonine. The next step arose when homoserine became exhausted from the environment, and an enzyme appeared that could convert aspartic semialdehyde to homoserine. In this manner the pathways evolved in a stepwise fashion.

The discovery of operons led Horowitz (1965) to extend his hypothesis to additionally take account of gene duplications as a source of new enzymes. Hegeman and Rosenberg (1970) suggested that regulation and the recruitment of activities from existing pathways may have been more important than gene duplication. Yčas (1974) and Jensen (1976) emphasized the importance of the broad specificity of early enzymes and the development of more specific enzymes by gene duplication followed by sequence divergence.

Surprisingly there seem to have been no attempts to examine the Horowitz hypothesis in terms of the prebiotic soup. The biosyntheses of threonine and methionine are rationally explained by this backwards stepwise development. The prebiotic synthesis of threonine is by a straightforward electric discharge reaction
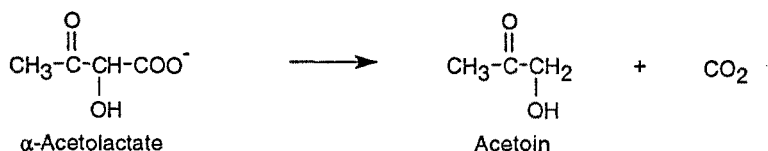
(Ring *et al.*, 1972). The prebiotic synthesis of methionine is from acrolein which also gives homoserine and homocysteine as well as glutamic acid and diamino butyric acid (Van Trump and Miller, 1972). Consistent with this picture is the homology of threonine synthase, threonine dehydratase and serine dehydratase (Parsot, 1986), and the homology of β-cystathionase and cystathionine δ-synthase of the methionine pathway (Belfaiza *et al.*, 1986).

The Horowitz hypothesis does not seem applicable to some biosynthetic pathways, e.g. the biosynthesis of purines from glycine. This pathway clearly cannot be based on components of the prebiotic soup since all the intermediates are ribosides which are unstable to hydrolysis. In addition, ribose is generally considered not to have been a significant component of the prebiotic soup (Shapiro, 1988). If the biosynthesis of purines was by the same pathway as at the present but without the ribose, the sequence would still contain mostly unstable compounds, for example $HCONHCH_2CONH_2$.

Branched-chain Amino Acid Biosynthesis

The contemporary biosyntheses of the branched-chain amino acids valine, isoleucine, and leucine by *Escherichia coli*, are shown in Figure 1. Four steps are common to all three synthetic pathways (Umbarger, 1987) and so the sequence of reactions are considered together. The pathways are the same in eubacteria, archaebacteria, and eukaryotes, and the enzymes are homologous (Xing and Whitman, 1991).

The Horowitz hypothesis cannot apply to the branched-chain amino acids valine, isoleucine and leucine, because their biosynthetic precursors are unstable, e.g.α-acetolactate decarboxylates readily because it is a β-keto acid, and so would not have been in the prebiotic soup. The half-life for decarboxylation is several days at room temperature (Hill *et al.*, 1979), and the anion decarboxylates readily on acidification:

$$CH_3-\underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{C}}-CH-COO^- \longrightarrow CH_3-\underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{C}}-CH_2 \;\; + \;\; CO_2$$

α-Acetolactate   Acetoin

In addition, acetolactate mutase catalyses an alkyl migration by an acyloin rearrangement which is almost unique in biochemistry, and so it may not be a very primitive enzyme.

A variation on the Horowitz proposal assumes that if a compound was present in the prebiotic environment, then so were its decomposition products. Consequentially, a biosynthetic pathway to this compound could have arisen in a stepwise fashion, utilizing the sequence of compounds available in the decomposition path-
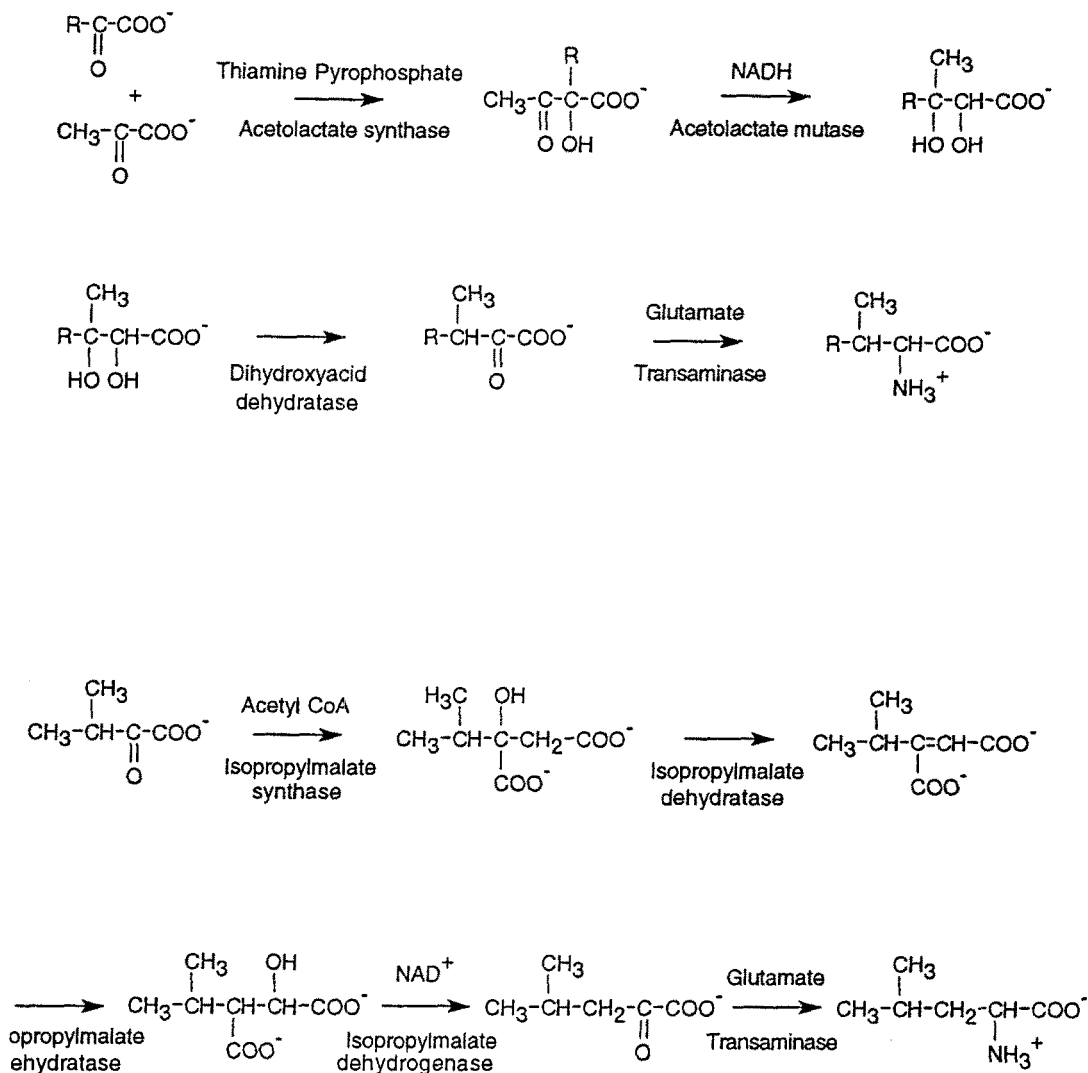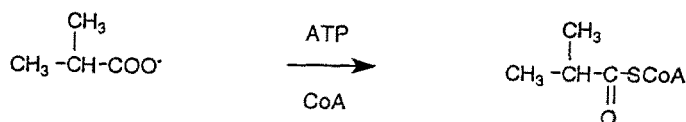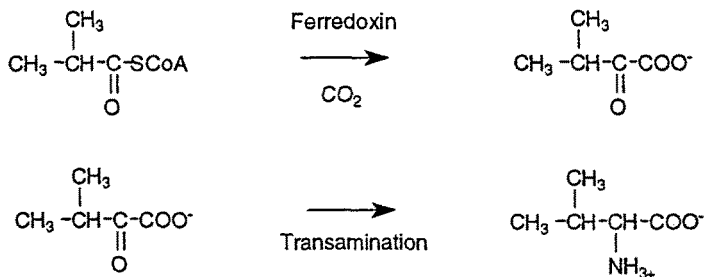
$$R-\underset{\underset{O}{\|}}{C}-COO^- \quad + \quad CH_3-\underset{\underset{O}{\|}}{C}-COO^- \xrightarrow[\text{Acetolactate synthase}]{\text{Thiamine Pyrophosphate}} CH_3-\underset{\underset{O}{\|}}{\overset{R}{C}}-\underset{OH}{\overset{}{C}}-COO^- \xrightarrow[\text{Acetolactate mutase}]{\text{NADH}} R-\underset{HO}{\overset{CH_3}{C}}-\underset{OH}{CH}-COO^-$$

$$R-\underset{HO}{\overset{CH_3}{\underset{OH}{C}}}-CH-COO^- \xrightarrow[\text{Dihydroxyacid dehydratase}]{} R-CH-\underset{\underset{O}{\|}}{\overset{CH_3}{C}}-COO^- \xrightarrow[\text{Transaminase}]{\text{Glutamate}} R-CH-\underset{NH_3^+}{\overset{CH_3}{CH}}-COO^-$$

$$CH_3-\underset{}{\overset{CH_3}{CH}}-\underset{\underset{O}{\|}}{C}-COO^- \xrightarrow[\text{Isopropylmalate synthase}]{\text{Acetyl CoA}} CH_3-\underset{}{\overset{H_3C}{CH}}-\underset{COO^-}{\overset{OH}{C}}-CH_2-COO^- \xrightarrow{\quad} CH_3-\underset{}{\overset{CH_3}{CH}}-\underset{COO^-}{C}=CH-COO^-$$

$$\xrightarrow[\text{opropylmalate ehydratase}]{} CH_3-\underset{}{\overset{CH_3}{CH}}-\underset{COO^-}{\overset{OH}{CH}}-CH-COO^- \xrightarrow[\text{Isopropylmalate dehydrogenase}]{\text{NAD}^+} CH_3-\overset{CH_3}{CH}-CH_2-\underset{\underset{O}{\|}}{C}-COO^- \xrightarrow[\text{Transaminase}]{\text{Glutamate}} CH_3-\overset{CH_3}{CH}-CH_2-\underset{NH_3^+}{CH}-COO^-$$

Fig. 1. Contemporary biosyntheses of the branched-chain amino acids. Valine, $R = CH_3$; Isoleucine $R = CH_2CH_3$.
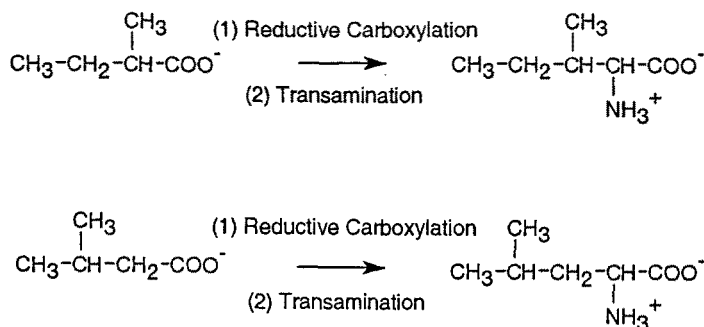
way, rather than the compound's precursors in the contemporary sequence. A modified reverse of the mammalian degradative pathway is, in the case of valine:

$$CH_3-\overset{CH_3}{CH}-COO^- \xrightarrow[\text{CoA}]{\text{ATP}} CH_3-\overset{CH_3}{CH}-\underset{\underset{O}{\|}}{C}-SCoA$$

$$CH_3-CH-C-SCoA \xrightarrow[CO_2]{Ferredoxin} CH_3-CH-C-COO^-$$

(with CH₃ substituents and O double bonds as shown)

$$CH_3-CH-C-COO^- \xrightarrow{Transamination} CH_3-CH-CH-COO^-$$

(with CH₃ and NH₃⁺ substituents as shown)

A similar degradative pathway is used in some anaerobic bacteria (Allison, 1978). It should be kept in mind that the acyl CoA could not have been in the prebiotic soup because it is unstable. The utilization of ATP and CoA in this pathway assumes that analogous enzymes were present, and that the enzymes using isobutyric acid arose by gene duplication.

The reactions giving isoleucine and leucine are shown below:

$$CH_3-CH_2-CH-COO^- \xrightarrow[(2)\ Transamination]{(1)\ Reductive\ Carboxylation} CH_3-CH_2-CH-CH-COO^-$$

(with CH₃ and NH₃⁺ substituents as shown)

$$CH_3-CH-CH_2-COO^- \xrightarrow[(2)\ Transamination]{(1)\ Reductive\ Carboxylation} CH_3-CH-CH_2-CH-COO^-$$

(with CH₃ and NH₃⁺ substituents as shown)

The reductive carboxylation is carried out by low potential ferredoxins, since NADH is not sufficiently reducing for this. Another possible reducing agent is pyrite (i.e., $FeS + H_2S \longrightarrow FeS_2 + H_2$) as proposed by Wächtershäuser (1988). This might work prebiotically or possibly as an early bacterial process.

The pathway discussed above is used by *Methanobacterium ruminantium, Bacteroides rumminicole* and other primitive prokaryotes in ruminants[*] for valine and isoleucine biosyntheses from isobutyric acid and α-methyl butyric acid, respectively (Robinson and Allison, 1969; Allison and Peel, 1971). The short chain aliphatic acids are likely to have been more abundant than the corresponding amino acids on the primitive Earth, as they are in the Murchison meteorite (Table I). A single enzyme could have produced valine, isoleucine and leucine from isobutyric, α-methylbutyric and isovaleric acids, respectively. When the fatty acid precursors were exhausted it would then have become necessary to develop the acetolactate pathway.

[*] The cow is modern, but the bacteria are ancient.

TABLE I

Carboxylic acids and corresponding amino acids occurring in the Murchison meteorite [data from Lawless and Yuen (1979) and Cronin and Pizzarello (1983)]

Carboxylic Acid	Abundance $nmolg^{-1}$	Corresponding amino acid	Abundance $nmolg^{-1}$
Ethanoic	1030	Alanine	44
Propanoic	1830	2-Aminobutyric	18
2-Methylpropanoic	500	Valine	10
Butanoic	380	Norvaline	3
2-Methylbutanoic	120	Isoleucine	4
3-Methylbutanoic	90	Leucine	4
Pentanoic	120	Norleucine	2
4-Methylpentanoic	70		
Hexanoic	60		
Heptanoic	30		
Octanoic	10		

The activation of the short chain fatty acids is a straightforward CoA synthesis which would have been mediated by an enzyme that could easily have been acquired by a gene duplication. The third step would involve a transamination enzyme that could also have arisen by gene duplication. The reductive carboxylation step is the only one requiring a new enzyme not easily obtained from a gene duplication, unless the Krebs cycle biosynthetic pathway is more ancient (see below). An alternative to the reductive carboxylation is to react acyl phosphate with formate (Tanaka and Johnson, 1971). This still requires the activation of the fatty acids, but the reaction may be simpler than the reductive carboxylation:

$$RCH_2-\overset{O}{\overset{\|}{C}}-OPO_3^{2-} \; + \; HCO_2^{-} \; \rightleftharpoons \; RCH_2-\overset{O}{\overset{\|}{C}}-COO^{-} \; + \; HPO_4^{2-}$$
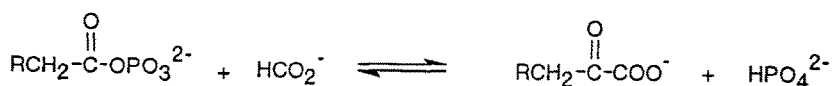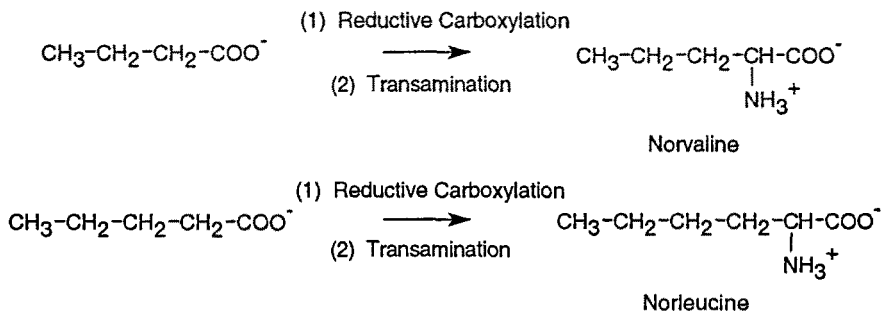
This sequence could be prebiotic. Transaminations are catalyzed non-enzymatically by pyridoxal (Metzler *et al.*, 1954), histidine (Doctor and Oré, 1969) and glyoxalate (Warren, 1971). The activation of the fatty acid would have been the result of the prebiotic activation reactions. A prebiotic version of the carboxylation could be as is shown below (Eggerer *et al.*, 1962):

$$CH_3-\overset{O}{\overset{\|}{C}}-SCoA \; + \; HCN \; \rightleftharpoons \; CH_3-\overset{O}{\overset{\|}{C}}-CN \; + \; CoASH$$
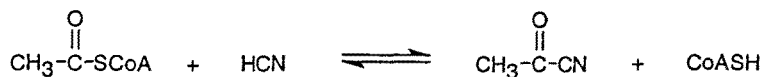
Acyl cyanides usually hydrolyze to the carboxylic acid and HCN except in strong acid, but soft nucleophiles such as H_2S can react under some conditions to give $CH_3COCSNH_2$ (Hünig and Schaller, 1982), which would hydrolyze to the keto acid. The prebiotic reaction conditions remain to be worked out.
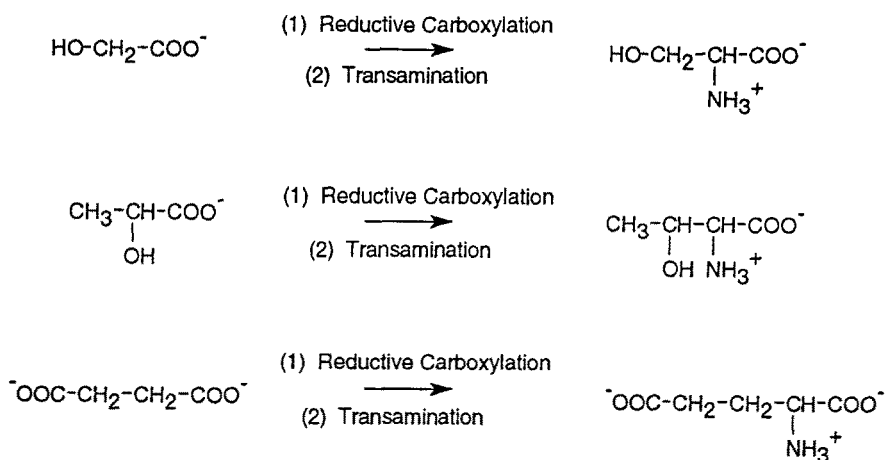
Fatty Acid Metabolism and the Absence of Norvaline and Norleucine from Proteins

If the fatty acid synthesis and degradative pathways developed early, then n-valeric, n-butyric and propionic acids would have been depleted early from the environment. This would have prevented the synthesis of norleucine, norvaline and α-amino-n-butyric acid by reductive carboxylation. This may explain the absence of these straight chain amino acids from proteins, which is otherwise difficult to account for (Weber and Miller, 1981):

$$CH_3-CH_2-CH_2-COO^- \quad \xrightarrow[\text{(2) Transamination}]{\text{(1) Reductive Carboxylation}} \quad CH_3-CH_2-CH_2-\underset{\underset{NH_3^+}{|}}{CH}-COO^-$$

Norvaline

$$CH_3-CH_2-CH_2-CH_2-COO^- \quad \xrightarrow[\text{(2) Transamination}]{\text{(1) Reductive Carboxylation}} \quad CH_3-CH_2-CH_2-CH_2-\underset{\underset{NH_3^+}{|}}{CH}-COO^-$$

Norleucine

Early Biosyntheses of Serine, Threonine and Glutamic Acid

Similar considerations may be applied to other amino acids with abundant prebiotic precursors. Thus serine could have been made from glycolic acid and threonine from lactic acid. These hydroxy acids are major products of prebiotic syntheses and also occur in the Murchison meteorite (Miller, 1957; Peltzer and Bada, 1978; Peltzer *et al.*, 1984). This scheme would have greatly increased the availability of serine and threonine, as glycolic and lactic acids are more stable than serine and threonine. A slight evolution of the reductive carboxylating enzyme would have allowed the synthesis of glutamic acid from succinic acid and alanine from acetic acid. This would have constituted the beginning of the reverse Krebs cycle used for amino acid synthesis in some anaerobic organisms (see below):

$$HO\text{-}CH_2\text{-}COO^-$$

(1) Reductive Carboxylation
⟶
(2) Transamination

$$HO\text{-}CH_2\text{-}\underset{\underset{NH_3^+}{|}}{CH}\text{-}COO^-$$

$$CH_3\text{-}\underset{\underset{OH}{|}}{CH}\text{-}COO^-$$

(1) Reductive Carboxylation
⟶
(2) Transamination

$$CH_3\text{-}\underset{\underset{OH}{|}}{CH}\text{-}\underset{\underset{NH_3^+}{|}}{CH}\text{-}COO^-$$

$$^-OOC\text{-}CH_2\text{-}CH_2\text{-}COO^-$$

(1) Reductive Carboxylation
⟶
(2) Transamination

$$^-OOC\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{NH_3^+}{|}}{CH}\text{-}COO^-$$

The first biosynthetic Pathway for the Aromatic Amino Acids

Prebiotic syntheses have been demonstrated for phenylalanine and tyrosine (Fried-mann and Miller, 1969), trytophan (Friedmann *et al.*, 1971) and histidine (Shen *et al.*, 1987, 1990). These are not particularly efficient syntheses, and the supply of these aromatic amino acids would have been quickly exhausted if they were components of early organisms. The reductive carboxylation/transamination scheme suggested here would have been a prebiotic source of these amino acids. Some primitive archaebacteria synthesize phenylalanine and tyrosine by this pathway (Sauer *et al.*, 1975). By a similar process tryptophan and histidine could be produced from indole acetic acid and imidazole acetic acid. The precursor acids are:
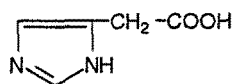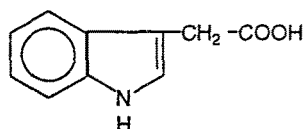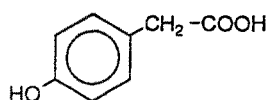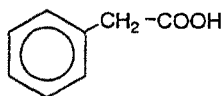
The efficient synthesis of these aromatic acetic acids may be prebiotic. In the

case of indole acetic acid the synthesis is shown below:
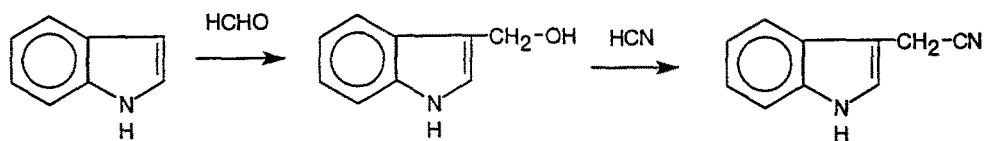
This is then followed by hydrolysis. A similar reaction should readily occur with phenol, but imidazole and especially benzene are relatively unreactive. Once these precursors were exhausted from the environment, the development of the complex shikimic acid aromatic biosynthetic pathway would have become necessary.

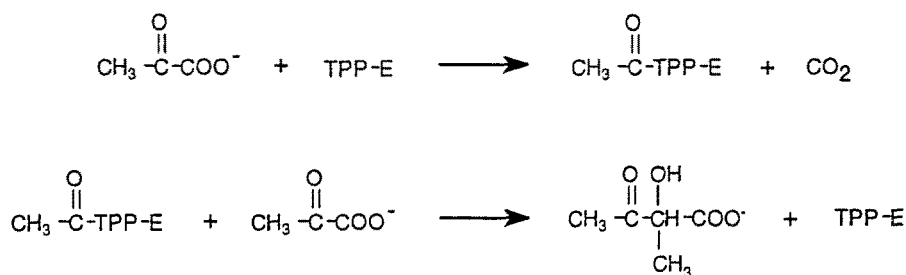## Origin of the Acetolactate Pathway

Although the branched chain fatty acids were more abundant than amino acids in the prebiotic soup, the short branched chain fatty acids quickly would have become exhausted. A reasonable order of development would be to assume that the leucine pathway from α-keto isovaleric acid developed first. The reaction of acetyl CoA with α-keto isovaleric acid is an aldol condensation for which the development of an anzyme, isopropyl malate synthase, may be easily envisioned.

Isopropyl malate isomerase (or dehydratase) catalyses a very similar reaction to that catalyzed by fumarase which proceeds non-enzymatically in acidic (Rozelle and Alberty, 1957), basic (Erikson and Alberty, 1959) and neutral (Bada and Miller, 1969) solutions at elevated temperature, and so development of the enzyme is easily envisaged. If fumarase or crotonase were present in the prokaryotic metabolic apparatus, the development of isopropyl malate dehydrogenase would have rapidly occurred by a gene duplication and subsequent sequence divergence. Isopropyl malate dehydrogenase is a standard NAD^+ alcohol dehydrogenase with a rapid non-enzymatic decarboxylation step.

Acetolactate synthase uses thiamine pyrophosphate. This reaction also occurs non-enzymatically with thiamine. The product is usually acetoin because the acetolactate rapidly decarboxylates (Breslow and McNelis, 1959).

Chang and Cronan (1988) demonstrated the functional and structural homology between pyruvate oxidase and acetohydroxy acid synthase in the branched chain amino acid biosynthetic pathway, and suggested that the synthase was derived from the pyruvate oxidase. The evolutionary conservation of the homology is easy to understand from the standpoint of the chemical reactions involved. Acetolactate synthase catalyzes the reactions

$$CH_3-\overset{\overset{\textstyle O}{\|}}{C}-COO^- \;+\; TPP\text{-}E \;\longrightarrow\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-TPP\text{-}E \;+\; CO_2$$

$$CH_3-\overset{\overset{\textstyle O}{\|}}{C}-TPP\text{-}E \;+\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-COO^- \;\longrightarrow\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-\overset{\overset{\textstyle OH}{|}}{\underset{\underset{\textstyle CH_3}{|}}{C}}H-COO^- \;+\; TPP\text{-}E$$

where TPP refers to thiamine pyrophosphate, E is the enzyme, and CH_3-CO-TPP-E is active acetaldehyde [CH_3-CO($^-$)] attached to the thiamine. Pyruvate oxidase catalyzes the reactions

$$CH_3-\overset{\overset{\textstyle O}{\|}}{C}-COO^- \;+\; TPP\text{-}E \;\longrightarrow\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-TPP\text{-}E \;+\; CO_2$$

$$CH_3-\overset{\overset{\textstyle O}{\|}}{C}-TPP\text{-}E \;+\; Fd_{ox} \;\longrightarrow\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-O^- \;+\; Fd_{red} \;+\; TPP\text{-}E$$
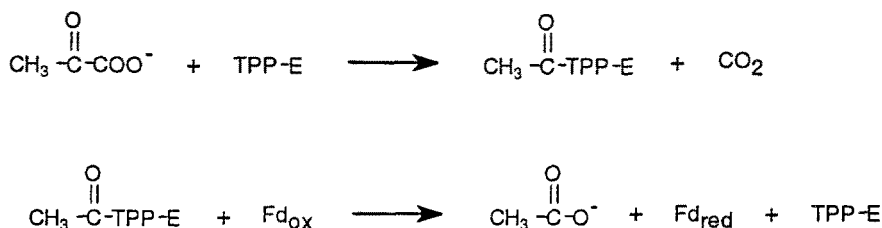
where Fd_{ox} and Fd_{red} are the oxidized and reduced forms of ferredoxin, but other electron acceptors such as NAD^+ and flavins are used with some enzymes. Other pyruvate oxidoreductases carry out the reaction with coenzyme A:

$$CH_3-\overset{\overset{\textstyle O}{\|}}{C}-TPP\text{-}E \;+\; CoA \;+\; Fd_{ox} \;\rightleftharpoons\; CH_3-\overset{\overset{\textstyle O}{\|}}{C}-SCoA \;+\; Fd_{red}$$

In this case the reaction may be reversible depending on the potential of the electron acceptor.

The homology of acetolactate synthase with pyruvate oxidase presumably extends mostly to the domains involved in the decarboxylation step, and not to other sections of the enzyme. We agree with the proposal of Chang and Cronan (1988) that acetolactate synthase was derived from pyruvate oxidase, but our modification to their scheme is that the pyruvate oxidase was a reversible enzyme operating in the keto acid synthesis direction rather than the irreversible direction. The acetolactate pathway would be completed with the development of the reductoisomerase and the dihydroxy acid dehydratase.

Transaminase

Pyruvate decarboxylase   Pyruvate   ⟶   Alanine

Fatty Acid Reductive Carboxylase

Aspartic acid

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-SCoA$$

Transaminase

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ HO-C-COO^- \\ | \\ CH_2 \\ | \\ COO^- \end{array}$$
Citrate

Aconitase
Isopropyl malate dehydrase

Citrate Synthetase
Isopropyl malate synthase

$$\begin{array}{c} COO^- \\ | \\ C=O \\ | \\ CH_2 \\ | \\ COO^- \end{array}$$
Oxaloacetate

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ C-COO^- \\ \| \\ HC \\ | \\ COO^- \end{array}$$
cis-Aconitate

Aconitase
Isopropyl malate dehydrase

Malate dehydrogenase
Isopropyl malate dehydrogenase

$$\begin{array}{c} COO^- \\ | \\ HO-CH \\ | \\ CH_2 \\ | \\ COO^- \end{array}$$
Malate

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CH-COO^- \\ | \\ HO-CH \\ | \\ COO^- \end{array}$$
Isocitrate

Isocitrate dehydrogenase
Isopropyl malate dehydrogenase

Fumarase
Isopropyl malate dehydrase

$$\begin{array}{c} COO^- \\ | \\ CH \\ \| \\ HC \\ | \\ COO^- \end{array}$$
Fumarate

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COO^- \end{array}$$
Succinate

Succinate dehydrogenase

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ C=O \\ | \\ COO^- \end{array}$$
α-Ketoglutarate

α-Ketoglutarate dehydrogenase
Fatty Acid Reductive Carboxylase
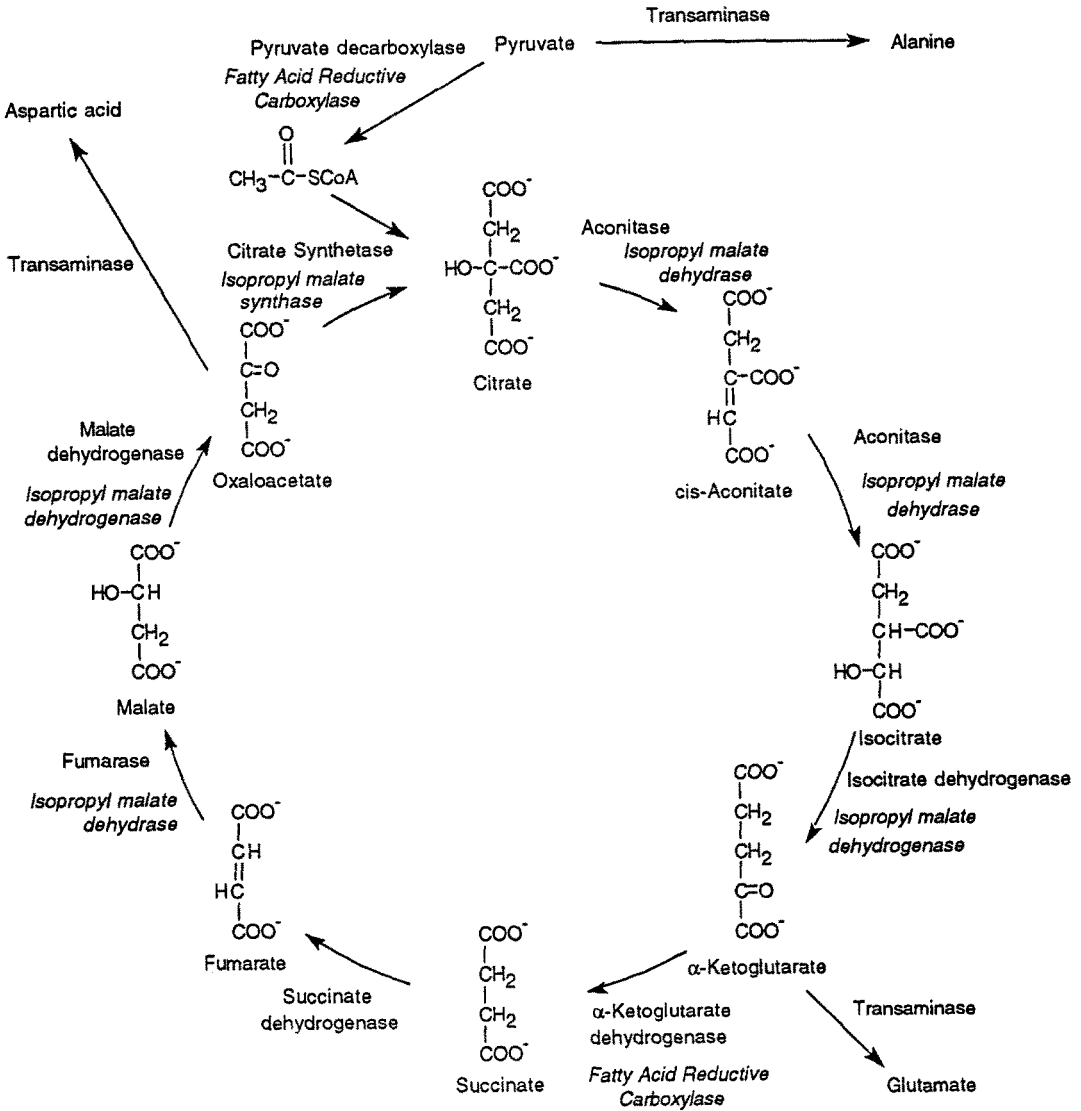
Transaminase

Glutamate

Fig. 2. The Krebs cycle. Also included in italics are the enzymes of the biosynthesis of leucine from α-ketoisovaleric acid and of the branched chain fatty acids to the keto acid (fatty acid reductive carboxylase).

Origin of the Krebs Cycle

The central role of the Krebs cycle in metabolism suggests that it is a very ancient pathway. Its origin is generally accepted as being for the biosynthesis of amino acids rather than ATP production, since no ATP is produced in the absence of an electron acceptor for the NADH produced in the cycle.

It is tempting to suggest that the Krebs cycle came before branched-chain amino acid biosynthesis because most of the twenty protein amino acids are derived from Krebs cycle intermediates. However, the Krebs cycle amino acids are among the most abundant prebiotic amino acids, i.e. alanine, aspartic acid and glutamic acid, and so they would have been depleted later than the less abundant branched-chain amino acids.

In contrast we propose that the Krebs cycle was developed by modification of the leucine biosynthetic pathway from valine. It is assumed that isopropyl malate dehydrogenase, isopropyl malate dehydratase and isopropyl malate synthetase were available, as well as the fatty acid reductive carboxylases and transaminase from the early branched-chain amino acid biosynthetic scheme. Thus only one new enzyme, succinate dehydrogenase, was needed to complete the Krebs cycle. The scheme is shown in Figure 2. The oxidative version of the Krebs cycle would have been established when sufficiently high potential electron acceptors (e.g. O_2) became available.

The counter argument can be made that the biosynthetic Krebs cycle came first and that the branched-chain amino acid pathways were developed from the Krebs cycle enzymes. This would be justified if the branched-chain amino acids were incorporated late into proteins, and the depletion of alanine, aspartic acid, glutamic acid, and related amino acids occured prior to the exhaustion of the branched-chain amino acids from the primitive ocean.

There have been a number of discussions suggesting that the origin of metabolism can be inferred from the backwards extrapolation of the contemporary pathways. Our proposals suggest the present pathways may have replaced even older biosyntheses. Some of the oldest pathways and their enzymes have survived in unusual organisms, but some may have been lost from biology in the same way that the precursor to RNA has disappeared.

Acknowledgments

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