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Perspectives in Amino Acid and Protein Geochemistry

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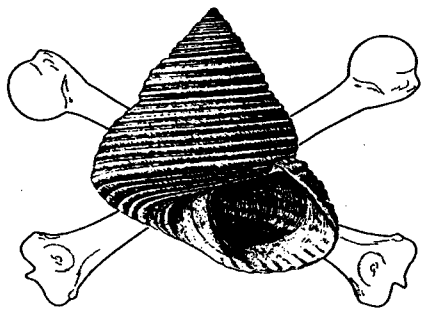

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4. Early pre- and post-biotic synthesis of alanine: an alternative to the Strecker synthesis

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The prebiotic synthesis of amino acids is considered a primary requirement for the origin of life. Much of the research in the origin-of-life field has therefore concentrated upon experiments designed to synthesize amino acids, either via electrical discharges (Miller, 1955), ultraviolet irradiation of solutions (Oparin, 1957), polymerization reactions under aqueous conditions (Peltzer et al., 1984; Stribling and Miller, 1987; Weber, 1998), or by Fischer Tropsch chemistry (Hayatsu and Anders, 1981). Although some success has been attained by each of these approaches, none has proved capable of synthesizing all of the 20 amino acids commonly employed by organisms, and most require conditions inhospitable to life. The most successful synthesis pathways involve the reaction and polymerization of aldehydes and cyanides (e.g., Strecker synthesis):

$$\mathrm{HCN} + \mathrm{CH_2O} \rightarrow \text{Amino acids} + \text{other nitrogen-containing organics.} \quad (4\text{-}1)$$

This reaction is capable of producing many nitrogen-containing compounds, including amino acids and nucleic acid subunits. The reaction is not, however, selective for specific amino or nucleic acid molecules. Furthermore, the presence of high concentrations of HCN, a powerful and highly reactive polymerizing agent, would seem to preclude the use of this pathway, by the earliest life-forms to produce amino acids. This is because HCN would react with, and ultimately destroy, the very catalysts necessary for maintenance of biochemical cycles. Thus, the fundamental question of how amino acids were synthesized by early life-forms remains unanswered.

Modern organisms employ specific enzyme-mediated biochemical pathways to control chemical reactions. The formation of amino acids proceeds via the reductive amination of α-keto acids produced initially in the citric acid cycle or elsewhere (e.g., α-ketoglutaric acid, pyruvic acid), or by modification of existing amino acids (e.g., cysteine from alanine). The enzymatic pathways are highly regulated to prevent wasteful production of unnecessary amino acids. The production of amino acids from α-keto acids involves the transfer of ammonia to the terminal carboxyl group of glutamate to form glutamine, followed by the amination and subsequent reduction of an α-keto acid to form the amino acid:

$$\mathrm{NH_3} + \text{glutamic acid} \rightarrow \text{glutamine:}$$
$$\text{glutamine} + \text{pyruvic acid} \rightarrow \text{glutamic acid} + \text{alanine.} \quad (4\text{-}2)$$

In some organisms (e.g., *Escherichia coli*) under ammonium-replete conditions, this reaction pathway is less complex. Under these circumstances, the glutamate/glutamine intermediate step is bypassed, and the α-keto acid is aminated and reduced in one step:

$$\mathrm{NH_3} + \text{pyruvic acid} \rightarrow \text{alanine.} \quad (4\text{-}3)$$

Of interest from a prebiotic chemistry standpoint is the observation that this reaction can take place in the absence of enzymes or external catalysts (Morowitz, 1992; Morowitz et al. 1996). It could be postulated that some of the biochemical pathways used by modern organisms were present in the first organisms (Morowitz, 1992). It follows logically that those reactions requiring little or no assistance from heterogeneous catalysts or enzymes and which produce a limited or controlled subset of possible reaction products are the most likely candidates for "relict" biochemical pathway status. The formation of alanine from pyruvic acid appears to meet these criteria, but this chemistry has been explored only under a very limited subset of conditions.

The goals of this work were to extend the conditions under which this reaction occurs in order to understand alanine synthesis and to understand the potential significance of this chemistry to prebiotic systems. Thus a series of experiments were undertaken with pyruvic acid, the simplest α-keto acid, and ammonium ions in aqueous solution under a variety of pH, temperature and pressure conditions.

MATERIALS AND METHODS

Samples were prepared from reagent-quality pyruvic acid and ammonium chloride. Most experiments were conducted with a solution containing 0.4 g pyruvic acid (purim grade; Fluka Chemical) and 1.0 g of a 4.7 M NH_4Cl solution. The pH of the solution was subsequently adjusted using a saturated solution of NaOH to the desired value, and finally all samples had distilled, deionized water added so that the total of NaOH solution plus water equaled 1.5 g. Dilution experiments were undertaken in the same manner, except that the volume of NH_4Cl solution and distilled, deionized water was increased to maintain NH_4^+ and pH conditions similar to those of the other solutions. Following initial sample preparation, 15 mg sample aliquots were placed in 10 mm (length) by 2 mm (diameter) gold (99.95% purity) tubes. The gold tubing was cleaned by sequential washes in boiling 10% HNO_3 and 10% HCl solutions, followed by distilled water washes and annealing at 500°C for 1 hr. One tube end was sealed by arc-welding prior to sample introduction. After sample introduction, solutions were placed in a glove bag and purged with N_2 for 20 min. The open tube ends were then crimp-sealed under N_2; this was followed by immersion in liquid N_2 and subsequent arc-welding of the tube end.

Atmospheric pressure experiments were conducted by placing the gold sample tubes within a larger glass tube (12 mm i.d. × 15 cm length) containing 0.05 ml H_2O to maintain internal pressure. The glass tube was sealed and the sample was placed in a convection oven for incubation. High-pressure experiments were conducted using an internally heated gas-pressure device (Yoder, 1950). Variations in temperature and pressure were controlled to better than 1°C and 101 kPa precision, respectively.

After incubation, sample tubes were washed in methanol, weighed, frozen in liquid N_2 and opened while frozen. Sample tube contents were extracted in either 1 ml methanol or 1 ml pH 2.0 HCl solution, followed by vortexing of sample contents for 30 seconds. Aliquots of sample were removed for both high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC–MS). Amino acid concentrations were determined using ion-exchange HPLC with post-column *o*-phthalaldehyde (OPA) derivitization (Serban et al., 1988). Mixed amino acid standards were also analyzed during each run. Typical uncertainties in yields were 5%, primarily because of errors in sample extraction and dilution. Sample aliquots for GC–MS analysis were derivitized using an acetyl chloride/isopropyl alcohol derivitization for the acidic moiety, followed by *N*-trifluoroacetic acid (*N*-TFA) derivitization for the amine moiety (Goodfriend 1991). Compounds were identified by GC–MS using a Hewlett Packard 6890 GC–MS system; either an HP-5 or a Supelco 1790 capillary column was used during experimental analysis.

RESULTS

Alanine synthesis

The formation of alanine from pyruvic acid requires both the reaction of the α-keto moiety with ammonia to form an intermediate, presumably an imine, followed by the reduction of that intermediate to form alanine. It is well known that acidic conditions promote decarboxylation reactions, which would increase the efficiency of pyruvic acid as a reducing agent. Previous work on alanine formation from pyruvic acid (Morowitz, 1992) has employed formic acid as a putative reducing agent, but it is clear from this work that no external reducing agent need be applied to the system. It is remarkable, however, how efficiently the reducing power is transferred to the imine intermediate at low pH values (figure 4-1). When one considers that the stoichiometry of the reaction is as follows:

$$2 \text{ pyruvic acid} + 1\ NH_4^+ \rightarrow 1 \text{ alanine} + 1 \text{ acetic acid} + H_2CO_3, \quad (4\text{-}4)$$

well over 50% of the pyruvic acid in these systems either ends up as alanine or is used as a reducing agent.

The synthesis of alanine from mixtures of pyruvic acid and ammonia was also rapid, reaching peak values at 100°C within 6–24 hr (figure 4-1). Synthesis was most rapid and efficient at pH values

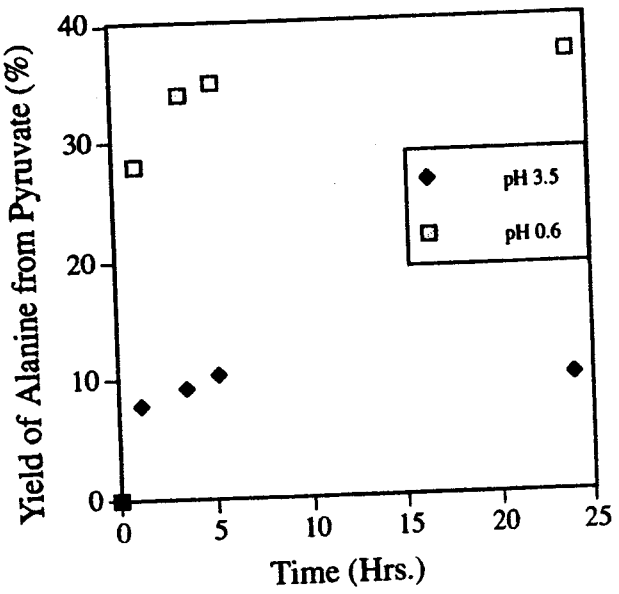

Fig. 4-1. Yield of alanine from pyruvic acid vs. time. Reaction conditions were 100°C and 0.1 MPa.

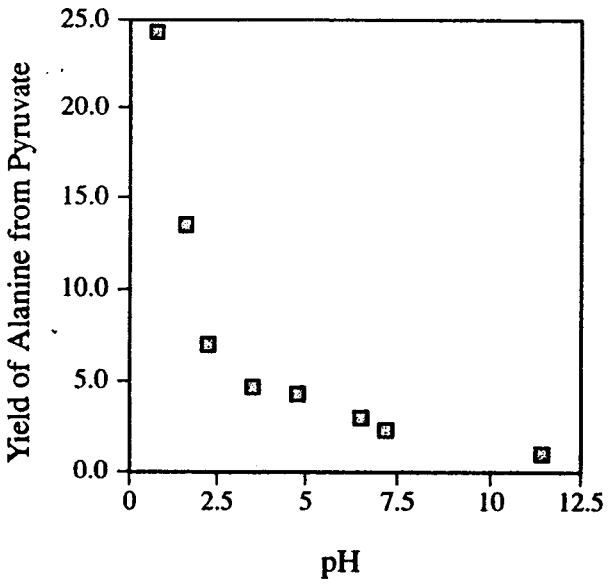

Fig. 4-2. Yield of alanine from pyruvic acid vs. pH. Reaction conditions were 100°C, 24 hr and 0.1 MPa.

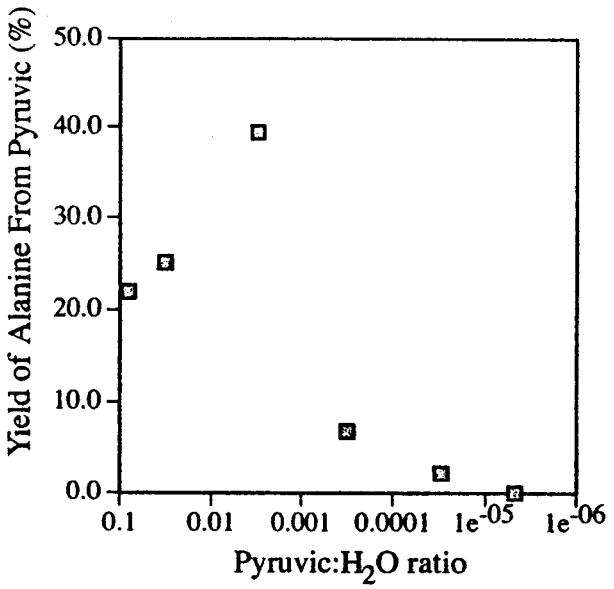

Fig. 4-4. Yield of alanine from pyuvic acid under different dilution conditions. Reaction conditions were 100°C, 24 hr, 0.1 MPa, 4.7 molar HN_4Cl and pH 0.7. Note: a 0.1 pyruvic acid:water ratio is equal to a concentration of 5.5 molar.

below 2, with conversion efficiencies of up to 40%. However, alanine concentrations plateaued after 6 hr.

The pH dependence of alanine synthesis is shown in more detail in figure 4-2. The reductive amination of pyruvic acid is very pH sensitive, with the highest conversion efficiencies being at pH 0.6–0.8 and dropping to below 10% above pH 2. No production of other identifiable amino acids was observed in the reaction mixtures. Experiments run at several pressures (figure 4-3) produced similar yields, with the exception that yields at pH = 2.95 were increased at the highest pressure.

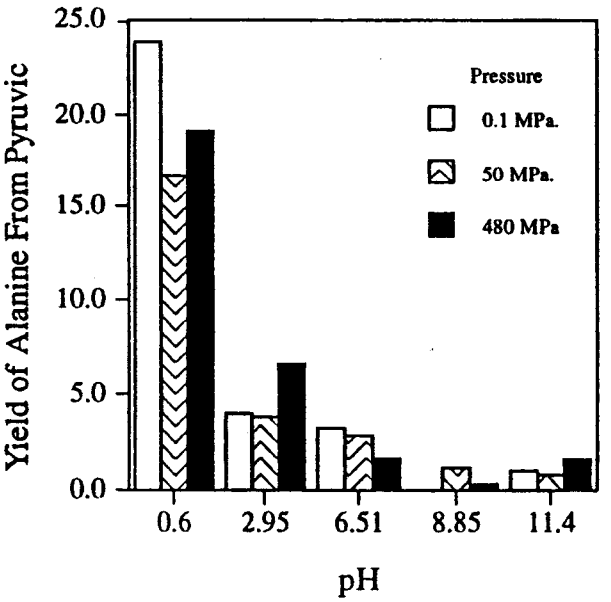

Fig. 4-3. Effect of pressure upon yields of alanine from pyruvic acid vs. pH. Reaction conditions were 100°C and 24 hr.

The influence of dilution of pyruvic acid upon alanine synthesis is shown in figure 4-4. Alanine yields increased slightly with increasing dilution until a pyruvic acid:water ratio of 1:100 (0.55 M) pyruvic acid was reached, at which point a sharp decline in yields was noted. Peak conversions (39%) of alanine were noted at pyruvic acid H_2O ratios of 1:100 .

GC–MS analyses

The efficiency of alanine synthesis at low pH values is also indicated by the lack of identifiable compounds other than alanine in the GC–MS trace (figure 4-5). However, the distribution of identifiable products from the reaction of pyruvic acid with ammonia shifted significantly with pH (figure 4-5). Below pH 2, the reaction products analyzable by GC–MS using isopropyl/*N*-TFA derivitization consisted entirely of alanine. However, above pH 2 another suite of products was observed in addition to alanine. Two major products, one eluting just after alanine at 12 min and the other at 15 min, were noted. Above pH 4, the predominant products shifted from alanine and smaller compounds to larger aromatic compounds, with one compound at 24 min dominating the system between pH 4 and pH 8.5. Above pH 8.5, the product mixture became more complex, with multiple compounds of aromatic character being observed.

The production of alanine is less efficient and the system becomes more complex as the pH increases. By pH 2.95, alanine comprised only 24% of the major products (by area). The two significant peaks, labeled Product A and Product B on figure

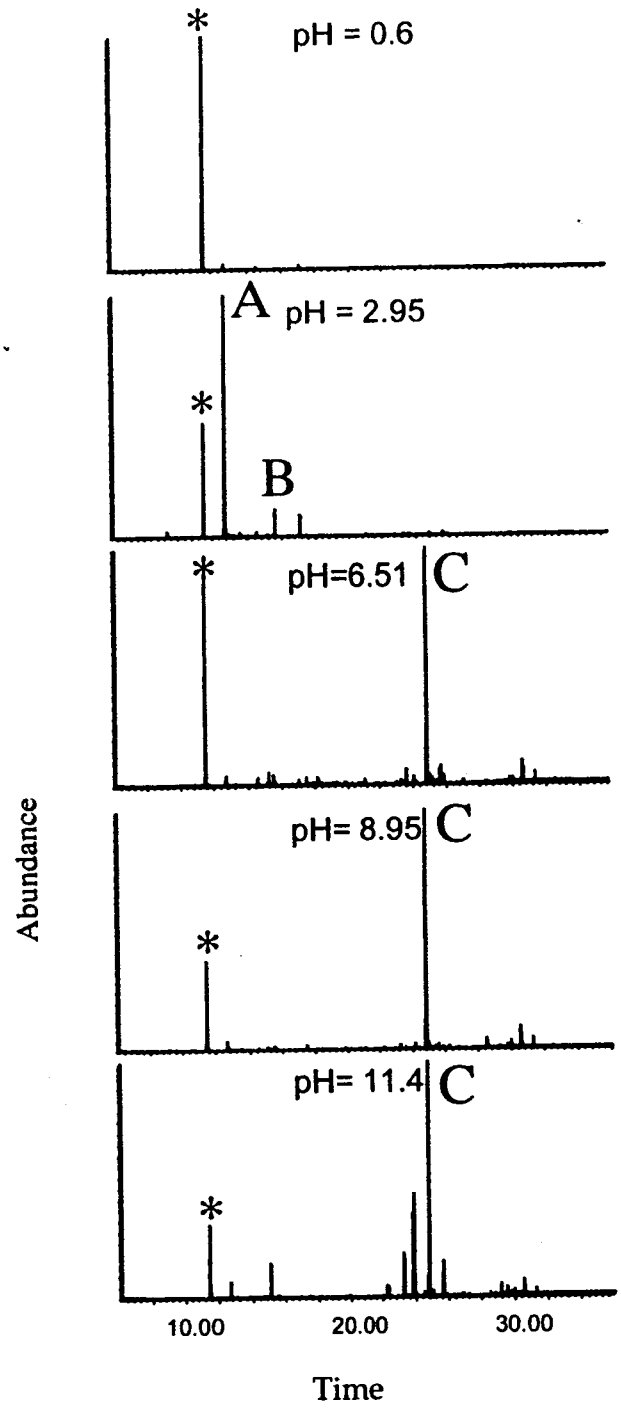

Fig. 4-5. Gas chromatography data from derivatized run products at different pH values. Reaction conditions: 100°C, 24 hr, and 50 MPa. The peaks representing alanine are denoted by asterisks; reaction products A, B and C are discussed in the text.

4-5, were identified (by fragmentation pattern) as aminated aldol condensation products. A proposed reaction scheme is shown in figure 4-6. Our work with pyruvic acid/water mixtures (G. D. Cody, unpublished data) has indicated that at intermediate pH values a primary reaction pathway for pyruvic acid is via aldol condensation. Once the aldol condensate is formed, the product is attacked either by ammonia or by another amine or amide. If attacked by ammonia, an imine is formed, which would then require reduction to the amine form to create a stable product. Also, the amine product can be attacked by acetic acid, which is present in high concentrations because of the decarboxylation of pyruvic acid, and this group may derivatize the amine as well (figure 4-6). This reaction pathway explains the lack, in Product A, of an $m/z = 69$ peak (expected from derivitization of the amine group by TFA), as well as a similarity of Product B's fragmentation pattern to that of alanine. Also these compounds or their precursors would be expected to hydrolyze to form alanine as well as other products (Streitwieser and Heathcock, 1985). The presence of bound (or otherwise underivatizable) alanine was tested by hydrolyzing an aliquot of the pH 2.95-run products in 6 N HCl for 15 min at 150°C. There was significantly more alanine present after hydrolysis (figure 4-7), supporting the hypothesis that products A and B were reduced aminated aldol condensates.

Further evidence for hydrolyzable products was obtained by examining the pressure dependence of alanine yields and reaction run products. The influence of pressure on the pyruvic acid/ammonia system was determined by comparing run products at 0.1, 50, and 480 MPa (figure 4-3). The general trend of decreasing yields above pH 0.6 was noted at all pressures, but at higher pressures the yield curve flattened out, with less production at the lowest pH and more observable alanine production at pH 2.95. It has been observed that the hydrolysis of protein bonds is accelerated by pressure (Qian et al., 1993); thus the increased yield at 480 MPa compared to lower pressures may be explained by hydrolysis of bound or underivatizable (by OPA) amino acid from compounds such as products A and B. Examination of the GC trace at pH 2.95 (figure 4-8) indicated that the yields of production compounds A and B were greatly reduced by increased pressure—a trend not noted in analysis of the other pH run products at pressure. It should be noted that overall yields, including the hydrolyzable fraction, were still greater in the high-pressure run than in low-pressure runs.

The pH 6.5 run again indicated a strongly pH-dependent change in the chemistry. Although alanine was still formed in significant quantity (figure 4-1), the primary product (Product C) was one with a much longer retention time, and the products noted in the pH 2.95 run were reduced to trace quantities. Product C was most closely related, in terms of mass spectra, to a family of nitrogen-containing aromatic carboxylic acids. We have observed the formation of aromatic compounds in the pyruvic acid/H_2O system as well (Cody et al., in press). The mass spectra from this compound indicated that it contained a nitro-

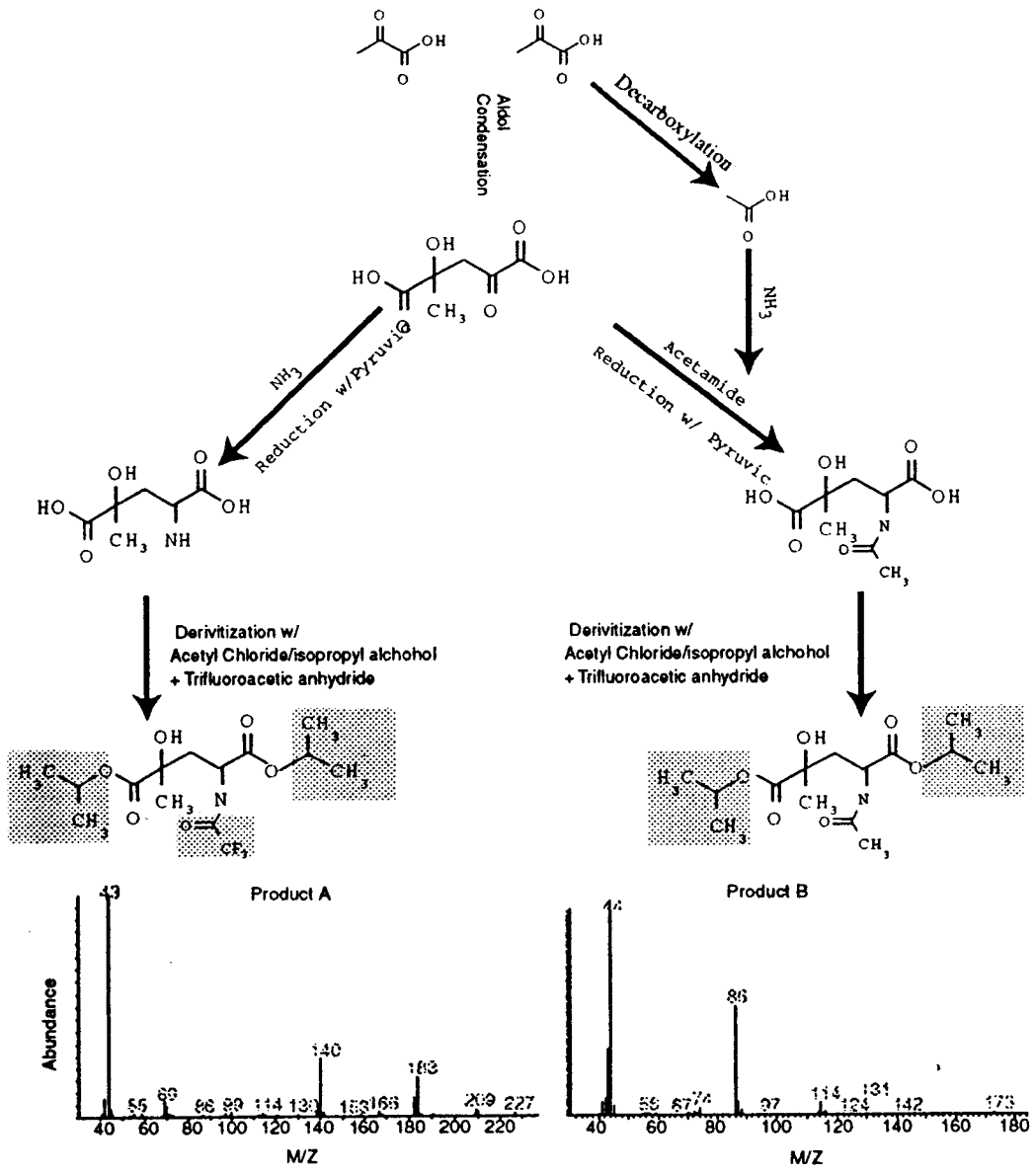

Fig. 4-6. Proposed reaction scheme and mass spectra for products "A" and "B" at pH 2.95. Shaded boxes represent functional groups added during derivatization.

gen moiety of either a derivatized primary amine (similar to Product A, and again lacking an $m/z = 69$ peak) or a 2 or 3° amine.

DISCUSSION AND CONCLUSIONS

Most prebiotic studies have concentrated upon the synthesis of compounds from simple precursors. Although the synthesis of ammonia in hydrothermal systems has been demonstrated (Brandes et al., 1998), the synthesis of pyruvic acid from simple one- and two-carbon precursors has not, to our knowledge, been reported. One hypothesis suggests that current biochemical pathways represent biochemical pathways of the earliest organisms, i.e., that core biochemical pathways have not been altered since the first life forms (Morowitz, 1992). If one accepts this hypothesis, then a reasonable corollary is that the chemical reactions that mimic biochemical pathways and that require the least specialized catalysis were the first to be utilized by life-forms. The results shown here indicate that the formation of amino acids from pyruvic acid and other α-keto acids fulfill this requirement. At low pH values, the synthesis of alanine is rapid, efficient and produces a minimum of side-products. Other proposed mechanisms employing cyano-containing compounds produce amino acids, but also produce β-amino acids as well as non-biological amines and acids (Stribling and Miller, 1987; Schesinger and Miller, 1983). The selectivity of the α-keto reaction,

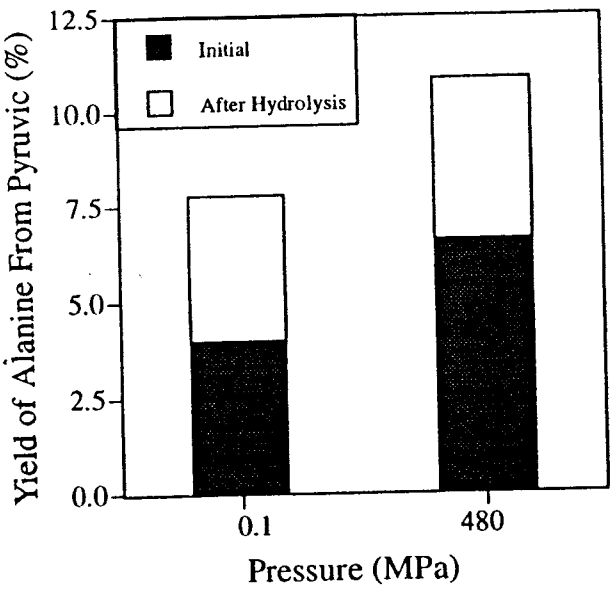

Fig. 4-7. Yield of alanine before and after hydrolysis at pH 2.95. Reaction conditions were 100°C and 24 hr.

from a biochemical perspective, is exactly what an early life form requires in order to target the production of valuable metabolic compounds. Given a source of α-keto acids, presumably from another primitive catalytic system, primitive life forms would have been able to produce pure α-amino acids relatively easily. Experiments with α-ketoglutarate and oxaloacetic acid have also shown that these compounds can produce specific amino acids as well, albeit in low yield (Morowitz et al., 1995; Maughan and Miller, 1999). Another possibility is the formation of α-keto acids via the decomposition of larger molecules (Cody et al., in press). Moreover, once formed, amino acids can act as nitrogen sources via transamination (Bishop et al., 1997). This work joins a body of research that suggests that the formation of amino acids in early biotic systems may have proceeded relatively easily provided that other pathways producing more reactive carbon compounds evolved first.

On the basis of the results in this paper, it is possible to envision a primitive metabolic pathway quite different than that utilized by modern organisms. By simple control of the acidity of the reactive site, a primitive organism could (in theory) produce longer chain aldol condensation products such as products A and B. The underivatized forms of these compounds bear a resemblance to glutamic acid. Given that we see evidence for the formation of peptide-like bonds between acetate and the amine group (products A and C), it is quite possible that this chemistry could, with slight modification, yield both larger metabolic products and simple peptide or peptide-like products. Aldol-condensation chemistry is quite different from the CO_2-addition chemistry undertaken by modern photosynthetic and chemoautotrophic organisms, and could represent an early alternative in the formation of larger molecules. At higher pH values, aromatic compounds could be formed that could (in theory) take the place of phenylalanine or form the basis for the formation of aromatic-containing compounds. All of these reaction pathways may also be influenced by the presence of mineral surfaces (Hafenbradl et al., 1995). As the true nature of prebiotic chemistry remains uncertain, the existence of alternative chemical pathways within the framework of biochemistry require exploration with the eventual goal of understanding the possibilities contained in present-day metabolic pathways.

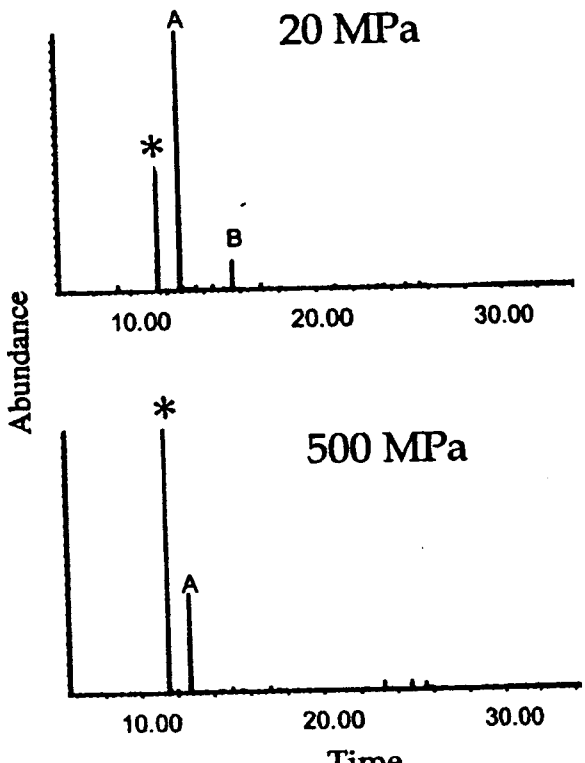

Fig. 4-8. Effect of pressure upon run products at pH 2.95 of pyruvic acid/ammonia reaction. Reaction conditions: 100°C and 24 hr.

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