CHEMICAL EVOLUTION OF THE CITRIC ACID CYCLE:
SUNLIGHT PHOTOLYSIS OF α-KETOGLUTARIC ACID

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Abstract. Sunlight photolysis of α-ketoglutaric acid produces succinic acid as a major product. Other higher molecular weight products are identified by GC–MS analysis. These results provide further support for the important role of succinic acid in chemical evolution.

1. Introduction

The chemical evolution of metabolism is a significant part of origin of life research (Buvet and LePort, 1973; Cairns–Smith and Walker, 1974; Hartman, 1975; Baldwin and Krebs, 1981), and of special interest is the origin and evolution of the citric acid cycle, the central pathway of energy metabolism in aerobic cells (Krebs, 1982; Gest, 1981). Despite this speculative attention, *experimental studies* in this area are rare. Thus, in one important series of papers, Ponnamperuma (1976, 1978, 1980, 1982) has shown that citric acid cycle carboxylic acids are produced upon ultraviolet and gamma irradiation of acetic, malic, or succinic acid solutions.

We have been interested in the question whether certain metabolic reactions have a propensity to occur non-enzymatically under mild primitive Earth conditions. If they do, one might be able to speculate that primitive life forms evolved around pre-existing chemical reactions (Gest, 1981) rather than the life forms developing catalysts to drive reactions that would otherwise not occur. Our initial experiments related to this question demonstrated the thermal conversion of citric acid to isocitric acid in a melt (140 °C) (Waddell and Osborne, 1982) via aconitic acid (Usol′tseva *et al.*, 1971).

Thus, our approach to chemical evolution studies is not the traditional one. We are not attempting polymerizations of products of primordial atmosphere experiments, nor are we concerned, at the present time, with possible advantages of discovered reactions to early life forms. What we do wish to accomplish in the initial stages of our work is to simplify, and to investigate only whether certain metabolic reactions can be coaxed to occur under mild conditions which might reasonably have been present on the primitive Earth (Waddell and Osborne, 1982).

The inclusion of molecular oxygen is valid in such experiments dealing with the Krebs citric acid cycle (CAC). An efficient CAC could only evolve after the O_2 content of the Earth's atmosphere increased to appreciable levels (Gest, 1981).

Therefore, we envision that O_2 was available from early photosynthetic metabolism and that the reactions and conditions described in this paper may have existed in time between the development of anaerobic photosynthetic life and the emergence of efficient aerobic cells. Indeed, we wish to point out that O_2 has been used in earlier chemical evolution experiments (Ponnamperuma, 1980).

The present paper discusses our attempts to induce (1) an aldol addition of acetic to oxalacetic acid to produce citric acid, (2) the addition of water to fumaric acid to yield malic acid, and (3) the oxidative decarboxylation of α-ketoglutaric acid to give succinic acid. Herein, we report that sunlight photolysis of α-ketoglutaric acid does produce succinic acid, a next metabolite in the modern CAC pathway. This novel transformation has not been previously reported in the literature.

2. Experimental Procedures

2.A. REAGENTS

α-Ketoglutaric and oxalacetic acids were crystallized from acetone-benzene 1:1. Fumaric acid was crystallized once from ethanol and once from acetone. Line distilled water was redistilled two times from $KMnO_4$. In preparing reagents and reaction solutions, standard precautions were taken to avoid bacterial contamination (see control experiment).

2.B. SUNLIGHT IRRADIATION

An oxygenated 0.1 M aqueous solution (10 mL) of purified α-ketoglutaric acid (acid form) in a quartz test tube was sealed and exposed to daily sunlight on the roof of the building for ten weeks during the Fall, 1985. After this period a 1.0 mL aliquot was removed and evaporated to dryness at room temperature for methylation and GC analysis. As a control, an identical sealed tube of the oxygenated 0.1 M α-ketoglutaric acid was set aside at the beginning of the experiment and kept in the dark at room temperature during the ten weeks.

2.C. GLASSWARE

All glassware was washed with concentrated HCl, rinsed with distilled water, and dried at 150 °C for one week.

2.D. ANALYSIS

Thin-layer chromatograms were obtained directly from the aqueous reaction mixture. Eastman silica gel sheets (0.25 mm thickness) were used and the chromatograms were developed with water-saturated ether-85% formic acid 7:1. Plates were visualized with a standard bromocresol green spray reagent for carboxylic acids (Beaudoin et al., 1973).

The solid residue from evaporation of the reaction mixture was methylated (CH_2N_2) and the esters were injected into a Carle analytical gas chromatograph

equipped with a 6 ft × ⅛ inch stainless steel column coated with 10% CS-10 on Chrom W−AW, 100/120 mesh. The column temperature was maintained at 152 °C. The carrier gas was helium with a flow rate of 15 mL per minute.
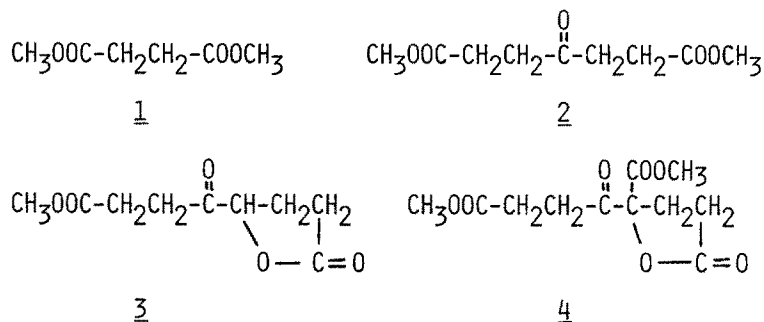
GC−MS analyses were carried out in the Laboratory of Chemistry, NIH, Bethesda, MD, on a LKB-Bromma 2091 instrument. The column was a HP ultraperformance capillary (25 m × 0.31 mm) packed with cross-linked methyl silicone. The temperature was programmed from 70° to 270° at 10°/minute. Dimethyl succinate was identified by direct comparison of its retention time and mass spectrum with an authentic sample. The structures of other photolysis product esters are discussed in Results and Discussion. The dark, room temperature control solution contained only α-ketoglutaric acid.
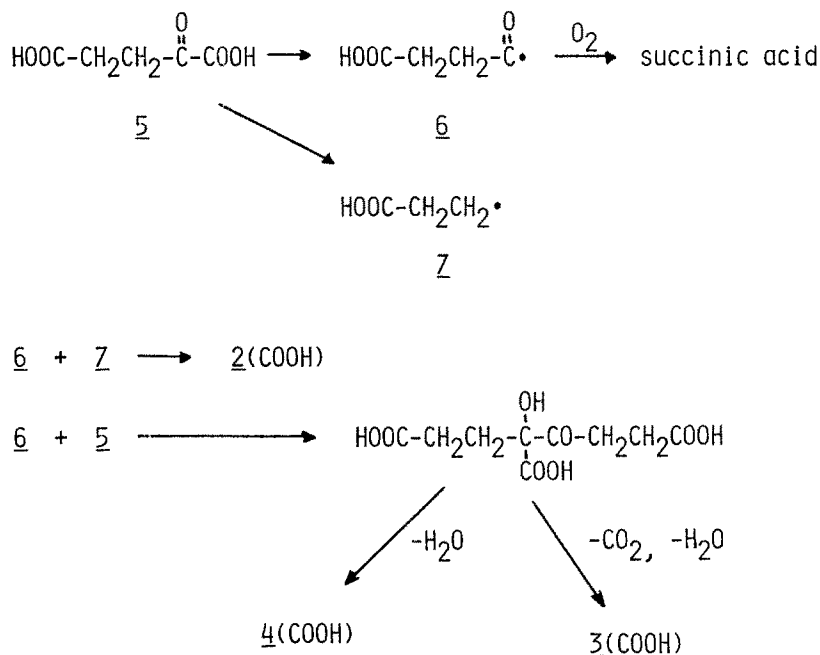
3. Results and Discussion

A 0.1 M aqueous solution of α-ketoglutaric acid in a quartz test tube was oxygenated, sealed, and exposed to sunlight for ten weeks, after which TLC analysis indicated the presence of succinic acid. Evaporation of an aliquot of this solution produced solid material which was methylated (CH_2N_2) and subjected to gas chromatography. The presence of dimethyl succinate was determined as the major product (ca. 25%) of a complex mixture. GC−MS analysis confirmed dimethyl succinate(*1*) and allowed identification of three higher molecular weight products (*2−4*). All of these products, *1−4* (carboxylic acids), can be viewed to arise from a single mechanism involving free radicals and intermediates that have been previously shown to form in the photolysis of α-keto acids (Steenken, 1975) (Scheme). Thus, Norrish type I cleavage of α-ketoglutaric acid(*5*) gives radicals *6* and *7*. Oxidation of *6* yields succinic acid while combination of *6* and *7* provides *2*(carboxylic acid). Addition of radical *6* to the ketone carbonyl of *5*(Steenken, 1975) followed by cyclization and decarboxylation gives *3* and *4*(COOH). Steenken *et al.* did not report on any organic products from their photolyses but only on the structures of radical intermediates.

The mass spectrum of product dimethyl succinate(*1*) was identical to that of authentic material. In general, a molecular ion is not present in the mass spectrum of dimethyl esters, but molecular weights are nevertheless reliably determined by observing an intense M-31(OCH_3) fragment ion (Howe and Williams, 1968). Product ester *2* shows an abundant fragment ion at m/z 171(M-31) along with the base peak at m/z 115($CH_3OOCCH_2CH_2CO\cdot^+$). An intense peak at m/z 87($CH_2CH_2COOCH_3$) confirms the structure. Analogously, the mass spectrum 0f *3*(MW = 200) shows m/z 169(M-31) along with 115 (base peak) and 85, representing the two halves of the molecule. Finally, product *4*(MW = 258) is confirmed by the following major mass spectral features: m/z 227(M-31), 199(M−$COOCH_3$), 115 (base peak), 143(M-115).

When a 0.1 M solution of α-ketoglutaric acid was *refluxed* (210 hours, over a period of 2.5 months) only recovered starting material was observed on TLC. Thus,

$$CH_3OOC-CH_2CH_2-COOCH_3$$

1

$$CH_3OOC-CH_2CH_2-\overset{\overset{O}{\|}}{C}-CH_2CH_2-COOCH_3$$

2

$$CH_3OOC-CH_2CH_2-\overset{\overset{O}{\|}}{C}-\underset{\underset{O-C=O}{|}}{CH}-CH_2CH_2$$

3

$$CH_3OOC-CH_2CH_2-\overset{\overset{O}{\|}}{C}-\underset{\underset{O-C=O}{|}}{\overset{\overset{COOCH_3}{|}}{C}}-CH_2CH_2$$

4

Scheme

$$HOOC-CH_2CH_2-\overset{\overset{O}{\|}}{C}-COOH \longrightarrow HOOC-CH_2CH_2-\overset{\overset{O}{\|}}{C}\cdot \xrightarrow{O_2} \text{succinic acid}$$

5   **6**

$$HOOC-CH_2CH_2\cdot$$

7

6 + **7** $\longrightarrow$ **2**(COOH)

6 + **5** $\longrightarrow$ $HOOC-CH_2CH_2-\underset{\underset{COOH}{|}}{\overset{\overset{OH}{|}}{C}}-CO-CH_2CH_2COOH$

$-H_2O$ $-CO_2, -H_2O$

4(COOH)   **3**(COOH)

the conversion of *5* to succinic acid is a photochemical and not a thermal process. Similarly, a sealed tube of oxygenated 0.1 M α-ketoglutaric acid was kept in the dark at room temperature for 10 weeks. Again, only recovered starting material was present. This demonstrates that neither bacterial contamination nor O_2 alone causes the reactions observed.

In other experiments, we attempted to effect a sunlight-induced addition of H_2O to fumaric acid to produce malic acid. However, after eight months of sunlight, the only product detected by TLC and careful GC analysis was the *cis* isomer maleic acid. In addition, an aqueous solution of purified acetic (0.1 M) and oxalacetic acid (0.1 M) was refluxed intermittently (200 hours over 2 months) in order to see if citric

acid could be produced by an aldol reaction. The product carboxylic acids (a complex mixture) were methylated and subjected to GC–MS analysis. Product esters were seen having molecular weights of 202, 214, 218, and 244, but no reasonable structures could be derived from the mass spectra obtained. Citric acid was not detected.

In conclusion, we have demonstrated the sunlight photolysis of α-ketoglutaric acid to succinic acid, a non-enzymatic reaction which mimics a step in the modern citric acid cycle. This chemical reaction may have occurred on the primitive Earth and been involved in the origin and evolution of the Krebs cycle pathway. In addition, our results provide further support for the important role played by succinic acid in chemical evolution (Ponnamperuma and Negron–Mendoza, 1982a).

Acknowledgments

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