γ-IRRADIATION OF MALIC ACID IN AQUEOUS SOLUTIONS

ALICIA NEGRON-MENDOZA*, REBECCA L. GRAFF, and CYRIL PONNAMPERUMA

Laboratory of Chemical Evolution, Department of Chemistry, University of Maryland, College Park, 20742, U.S.A.

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Abstract. The γ-irradiation of malic acid in aqueous solutions was studied under initially oxygenated and oxygen-free conditions in an attempt to determine the possible interconversion of malic acid into other carboxylic acids, specifically those associated with Krebs cycle. The effect of dose on product formation of the system was investigated. Gas-liquid chromatography combined with mass spectrometry was used as the principal means of identification of the non-volatile products. Thin layer chromotography and direct probe mass spectroscopy were also employed.

The findings show that a variety of carboxylic acids are formed, with malonic and succinic acids in greatest abundance. These products have all been identified as being formed in the γ-irradiation of acetic acid, suggesting a common intermediary. Since these molecules fit into a metabolic cycle, it is strongly suggestive that prebiotic pathways provided the basis for biological systems.

1. Introduction

Chemical evolution on Earth, assumed to have occurred some 3.5 to 4.6 billion years ago (Schopf, 1967; Tilton and Steiger, 1965), is believed to have resulted from subjecting the Earth's primitive atmosphere to various energy sources. Ultraviolet light from the sun, electric discharges in the form of lightning, heat from volcanoes and hot springs, and ionizing radiation from radionuclides are all possible agents for the formation of complex organic molecules from water, hydrogen, methane and ammonia, the proposed constituents of the Earth's primitive reducing atmosphere (Oparin, 1938).

Acetic acid has been identified as a product in simulated primitive Earth experiments (Miller, 1957; Allen and Ponnamperuma, 1967). From a series of experiments with glacial and aqueous acetic acid using the energetic radiation from γ-rays as the energy source, numerous di- and tricarboxylic acids were identified as being formed. Some of these were acids associated with the Krebs cycle (Negrón-Mendoza and Ponnamperuma, 1974).

Malic acid plays a dual role in chemical evolution. It acts not only as an intermediate in the Krebs cycle, but also is believed to be the prebiotic precursor of the pyrimidines (Miller, 1957; Negrón-Mendoza and Ponnamperuma, 1967).

Various investigators have studied the effect of ionizing radiation on aqueous solutions of dicarboxylic acids. From the irradiation of malonic and succinic acids with X-rays, Fricke *et al.* (1938) detected hydrogen. Carbon dioxide was also detected from alonic acid. Tanaka and Wang (1967) studied the γ-irradiation of succinic acid. By thin layer chromatography they found the production of malonic, malic, tricarballylic, oxalacetic, pyruvic acids, etc. In their malic acid study, malonic, oxalacetic, and pyruvic acids were

* Present address: Centro de Estudios Nucleares, Universidad Nacional Autónomá de México, Circuito Exterior, Cd. Universitaria, México 20, D.F. Mexico.

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reported as products. X-irradiation of malic acid was studied by Putney and Pratt (1956). They reported the formation of CO_2, oxalacetic (in oxygenated systems only), and pyruvic acids. Formation of polymeric products from malic acid irradiation has not been previously reported.

In the present study aqueous solutions of malic acid were γ-irradiated under oxygenated and oxygen-free conditions to determine both the effect of doses on product formation and the possible interconversion of malic acid into other Krebs cycle intermediates.

2. Experimental Procedure

A. PREPARATION OF SAMPLES AND IRRADIATION

Malic acid was obtained from Fisher Scientific, Inc. and purified through recrystallization from acetone and acetone/CCl_4. Triple-distilled water (Draganic *et al.*, 1971) was used in the preparation of the 0.1M aqueous malic acid solution. Prior to use, the stock solution was kept refrigerated in a tightly sealed volumetric flask. Duplicate solutions, 8 ml each of the 0.1M malic acid (pH 2.25), were transferred to 10 ml glass ampoules, then frozen and sealed. In the oxygen-free experiments, sealing was preceded by thrice outgassing and N_2 saturation, followed by evacuation to 7×10^{-3} mm of Hg using a vacuum line. In the oxygen containing experiments, the vessels were flushed with oxygen three times to ensure that the desired atmosphere was fully established.

The sealed equilibrated room-temperature ampoules were irradiated using a ^{60}Co irradiator at a dose rate of 1.77×10^{20} initially for periods varying from 0.5 to 17.0 hr. In the oxygenated system the doses 8.85×10^{9}, 35.2×10^{19}, 7.00×10^{20} and 1.03×10^{21} eV g^{-1} were used, while in the oxygen-free system the doses were 10.15×10^{19}, 67.6×10^{19}, 10.6×10^{20}, 11.8×10^{20}, 13.7×10^{20}, 18.2×10^{20} and 28.7×10^{20} eV g^{-1}. A Fricke dosimeter was employed to measure the dose rate with $G(Fe^{+3}) = 15.6$ and $=2197 M^{-1}$ cm^{-1} at 25 °C. Following irradiation and prior to analysis these samples were kept sealed and frozen.

B. ANALYSIS OF NON-VOLATILE PRODUCTS

Ampoules were broken and the irradiated solutions were divided into two fractions. Fraction I was esterified and analyzed by GC and GC/MS. Fraction II was converted into its 2,4-dinitrophenylhydrazone derivatives and subjected to thin layer chromatography (T.L.C.).

(1) Esterification, CS and GC/MS Analysis

Four ml from each ampoule was transferred to Pyrex^{R} culture tubes and freeze-dried. Methyl esters were prepared according to the procedure of Metcalf and Schmitz (1961). A known quantity of adipic acid was added and used as internal standard, before the esterification step. These esters were extracted into a total of 6 ml benzene and reduced to dryness under nitrogen. Hexane and methanol was then added to the culture tube and the esters were introduced into a G.C. An Antek 300 series gas chromatograph, pro-

grammed from 50 °C to 230 °C at 2 °C min^{-1} and employing FID, was used. The 10 ft steel column was packed with 8% Hi Eff 8 BP on acid washed Chromosorb W.

Following tentative identification through retention times, the sample and standards were subjected to GC/MS. A Perkin Elmer 900 gas chromatogram and a Dupont CED-21 492 mass spectrometer with silicon membrane separator was used. The separator was maintained at 200 °C. Chemical ionization studies were performed in a Finnigan GC/MS system, series 3200, in which a computer system was used to acquire, store and process the data.

(2) Analysis of Keto-acids by T.L.C.

1 ml of 2,4-dinitrophenylhydrazine solution (0.2% in 2N HCl) was added to 1 ml of the 0.1M irradiated solution and the mixture was refrigerated overnight. After extraction with ethyl acetate and reduction under nitrogen, these hydrazones along with standards were spotted on a silica gel 60 plate. $MeOH/CHCl_3$ (3:2 v/v) was used for development.

Confirmation of oxalacetic in the initially oxygenated system (whose identity could not be confirmed with GC/MS of the methyl esters due to its close proximity to malic acid ester) was obtained by comparison of the MS of both the standard 2,4-D.N.P. derivative and the spot of equivalent rf (rf = 0.56). This was accomplished by the scraping of the spots, extraction with ethyl acetate, reduction under N_2 and direct probe mass spectrometry.

3. Results

The following products were identified by GS and confirmed by GC/MS.

(a) *Initially oxygenated system*

glycolic acid, malonic acid, succinic acid, oxalacetic acid, tricarballylic acid, t-aconitic acid, citric acid, 1,2,3,4-butanetetracarboxylic acid, and carboxysuccinic acid.

(b) *Oxygen-free system*

malonic acid, succinic acid, tricarballylic acid, citric acid, 1,2,3,4-butanetetracarboxylic acid, and carboxysuccinic acid.

In the initially oxygenated system β-ketoadipic was identified by MS fragmentation pattern only, as the standard of this acid was not obtained. The same peak as the above was present in GC chromatograms of the oxygen-free system. A peak corresponding to t-aconitic acid was also located in the chromatograms of the oxygen-free system; however, the mass spectrum was not obtained.

Gas chromatograms of the methyl esters of products formed after four hours irradiation are shown in Figure 1. In the oxygen-free system four major products are formed. Three of these compounds correspond to malonic, succinic, and 1,2,3,4-butanetetracarboxylic acids. The fourth, by its MS, appears to have an M^- at 272 corresponding to either a $C_{12}H_{16}O_7$ or a $C_{13}H_{20}O_6$ compound.

In the initially oxygenated system malonic and succinic acids are the major products.

A study of the effect of dosage change on product formation was also undertaken.

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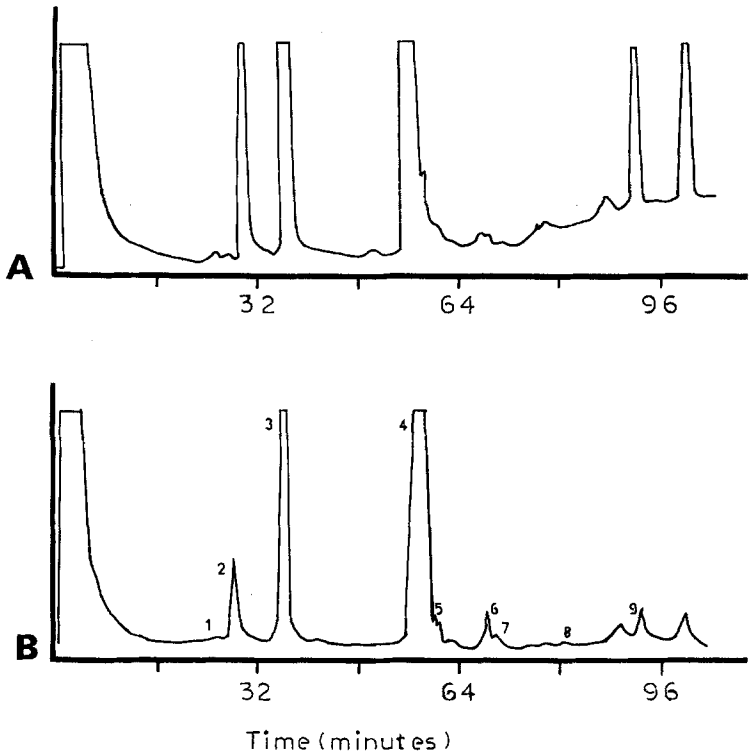

Fig. 1. Gas liquid chromatogram of the esterfied non-volatile carboxylic acids produced after irradiation of 0.1M malic acid. (A) In an oxygen-free atmosphere, with a radiation dose of 6.76×10^{20} eV g^{-1}. (B) In an initially oxygenated atmosphere with a radiation dose of 7×10^{20} eV g^{-1}. The numbered peaks correspond consecutively to glycolic, malonic, succinic, malic, oxalacetic, tricarballylic, taconitic, citric acid, 1,2,3,4-butanetetracarboxylic acids.

The butanetetracarboxylic acids begin to decline in quantity. Glycolic acid, present at 4 hr, is absent at 6 hr. A lack of available oxygen would explain this. Tricarballylic and t-aconitic appear to be more stable, as over the time span used, net decomposition was not observed.

A quantitative study of dosage effect on major products formed in the oxygen-free system was made. These results show that during a radiation period between 7 and 17 hr all major products undergo net decomposition. The G value for malic acid decomposition in oxygen-free system was 4.2 at 1.18×10^{21} eV ml^{-1}.

4. Discussion

The two fundamental effects of γ-irradiation are ionization and excitation of molecules. These species, through the modes of decomposition, rearrangement, and photon emission, dissipate energy to form free radicals. It is the further reaction of these free radicals that

gives rise to the products noted. Among the expected net reactions are polymerization, decomposition and isomerization.

In the dilute solutions used, it appears feasible to assume that the molecules of the solvent are directly affected by the γ-irradiation (Draganic *et al.*, 1971).

$$H_2O \xrightarrow{\sim} {}^{\circ}OH\ {}^{\circ}H,\ e^-_{ag},\ H_2,\ H_2O_2.$$

Solute reactions would then be due either to a secondary attack by solvent-free radicals or by a unimolecular decomposition. The graph of malic acid decomposition in the oxygen-free system as a function of the dose is shown in Figure 2. Complete decomposition is observed after 17 hr irradiation at 1.69×10^{20}. The black dots represent the decomposition in the presence of oxygen.

The purpose of this investigation was to study the interconversion of carboxylic acids related to biological processes by using gamma rays on malic acid. For this purpose, it was necessary to use a high radiation dose in the megarads region.

Gaseous products were formed, specially CO_2 as well as other carbon-containing compounds such as ketones. They, however, were not analyzed. Therefore, the material balance was not calculated, since accumulation of products was allowed, the radiolysis mechanism is uncertain. The suggestions are based on the radiolysis of similar compounds reported in the literature (Garrison *et al.*, 1953; Johnson *et al.*, 1953).

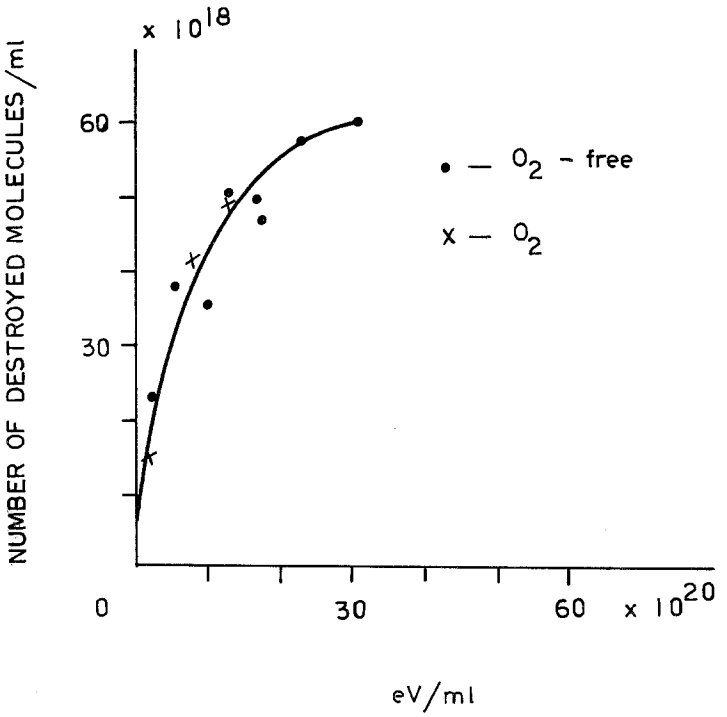

Fig. 2. Decomposition of 0.1M malic acid (oxygen-free) by γ-rays as function of dose. Dose rate 1.69×10^{20} eV ml^{-1} hr^{-1}.

Succinic acid was the most abundant radiolysis product from malic acid. Most of the other products can be explained as secondary products from the decomposition of succinic acid formed during the radiolysis in a manner similar to that proposed for acetic acid (Garrison, 1953).

Although oxygen is consumed rapidly, the mechanism of formation of the products in the oxygenated system may be related to the X-ray studies of lactic acid by Johnson *et al.* (1953), in which the initial process in the radiation-induced reaction may be assumed to be the dehydrogenation by an hydroxyl radical followed by direct addition of molecular oxygen.

$$HOOC(OH)CH{-}CH_2COOH + {}^{\bullet}OH \longrightarrow HOOC(OH)\dot{C}{-}CH_2COOH + H_2O$$

$$HOOC(OH)\dot{C}{-}CH_2COOH \xrightarrow{O_2} HOOC(OH)\underset{\displaystyle {}^{\bullet}O_2}{\overset{\displaystyle |}{C}}{-}CH_2{-}COOH \xrightarrow{{}^{\bullet}OH}$$

$$HOOC\underset{\displaystyle C}{\overset{\displaystyle ||}{C}}H_2COOH + H_2O + O_2$$

Glycollic acid may be formed by the following reaction: (Johnson *et al.*, 1953).

$${}^{\bullet}CH_2COOH + O_2 \longrightarrow {}^{\bullet}O_2CH_2COOH \xrightarrow{H_2O} CH_2(OH)COOH + HO_2^{\bullet}$$

$$2CH_2^{\bullet} \longrightarrow H_2O_2 + O_2$$

$$HO_2^{\bullet} \longrightarrow H_2O_2 + O_2$$

A comparison between the oxygenated and oxygen-free systems shows that oxygen is consumed immediately, and the decomposition of malic acid in both systems was identical. The effect of dose change on both systems changed the relative amounts of each product formed. It was noted that the chromatograms of the oxygenated and oxygen-free systems were of the same pattern, except for the presence of glycollic and oxalacetic in the first one.

5. Conclusion

Under the conditions employed, malic acid is a highly reactive species whose decomposition ultimately results in the formation of a variety of carboxylic acids. The acids formed have also been identified as products from the irradiation of acetic acid. This suggests that a common intermediary is involved.

Many of these acids are also of biological importance. Succinic, oxalacetic, and citric acids are intermediates in the Krebs cycle, while a malonic acid derivative is important in fatty acid biosynthesis. The results obtained therefore tend to further substantiate the proposal that irradiation on the primitive earth could have contributed to the formation of biologically relevant compounds prior to life itself. Since these compounds are intermediates in modern metabolic cycles, we may speculate that life could have utilized already existing molecular relationships.

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