J. Mol. Evol. 14, 235–241 (1979)

Journal of Molecular Evolution

Cyanamide Mediated Synthesis Under Plausible Primitive Earth Conditions

VI. The Synthesis of Glycerol and Glycerophosphates

D.E. Epps*, D.W. Nooner**, J. Eichberg***, E. Sherwood, and J. Oró

Departments of Biophysical Sciences and Chemistry, University of Houston, Houston, Texas 77004, U.S.A.

Summary. The formation of glycerol occurs when a solution of DL-glyceraldehyde is heated in the presence of hydrogen sulfide at room temperature. DL-glyceraldehyde and dihydroxyacetone treated with hydrazine, as well as DL-glyceraldehyde incubated with formaldehyde are also partially converted to glycerol. The yields of the above reactions are from approximately 1% to about 3%. The formation of glycerophosphates occurs when glycerol is heated with ammonium dihydrogen phosphate and either urea or cyanamide. The yield of glycerophosphates is about 30%, most of which is *sn*-glycero-1(3)-phosphate. These findings indicate that glycerol and *sn*-glycero-3-phosphate, which are moieties of glycerolipids, could have been formed under conditions which may have prevailed on the primitive Earth.

Key Words: Glycerol – Glycerophosphate – Cyanamide – Glycerolipids – Prebiotic synthesis

1. Introduction

One of the fundamental requirements for the evolution of the first cellular organism would be the formation of a plasma membrane. In present day organisms this membrane is composed of a phospholipid bilayer with associated protein. It is therefore of importance to understand the mechanism for the synthesis of phospholipids on the prebiotic Earth and how these molecules interacted with simple peptides to form a semipermeable vesicle. The primitive membranes of such vesicles would have protected the evolving macromolecules inside from adverse environmental influences while allowing limited access to the simple molecules being synthesized on the outside.

In our investigations of this critical phase of chemical evolution (Oró et al., 1978), we have shown how acylglycerols (Eichberg et al., 1977) and phosphatidic acids (Epps

*Present address: Hormel Institute, Austin, MN 55912, USA

**Present address: Spectrix Corp., Houston, Tx 77054, USA

***To whom correspondence should be addressed

0022–2844/79/0014/0235/$ 01.40

et al., 1978) may have been synthesized on the primitive Earth. At the time this work was done no one had reported on the abiotic synthesis of glycerol or glycerophosphate. In this paper we show that various prebiotic reducing agents including hydrogen sulfide, hydrazine and formaldehyde are capable of reducing DL-glyceraldehyde or dihydroxyacetone to glycerol and that glycerol is readily phosphorylated by well established abiotic phosphorylation procedures (Lohrmann and Orgel, 1971; Schwartz, 1972).

2. Experimental

2.1. Materials

Dihydroxyacetone (Sigma), DL-glyceraldehyde (Sigma), formalin (37% formaldehyde solution) (Fisher Scientific Co.), hydrazine (Eastman Chemicals), hydrogen sulfide (Air Specialty Products), Sigma-Sil-A (Sigma), glycerol kinase (Worthington), bovine serum albumin (Sigma), and ^{32}P-orthophosphoric acid (New England Nuclear) were used for synthesis and identification of glycerol. 2-^{14}C-glycerol (specific activity 14 μCi/μ mole, New England Nuclear), cyanamide (Eastman Chemicals), urea (Schwarz Mann) and imidazole (Eastman Chemicals) were used for the synthesis of glycerophosphates. Authentic cyclic glycerophosphate (*sn*-glycerol-2,3(1,2)-cyclic phosphate) was synthesized according to Lapidot et al. (1969). All solvents and chemicals were reagent grade. ^{32}P-ammonium dihydrogen phosphate was prepared by adding ^{32}P-phosphoric acid to a solution of monobasic ammonium phosphate and neutralizing with a stoichiometric amount of ammonium hydroxide.

2.2 Chromatographic Procedures

Aliquots (0.1–0.2 ml) of reaction products to be examined by gas chromatography and combined gas chromatography-mass spectrometry, were evaporated to dryness under nitrogen and the residue shaken with 0.3 ml Sigma-Sil-A (trimethylchlorosilane-hexamethyldisilazinepyridine 1:3:9) for 10 min at room temperature. The excess of derivatizing reagent was removed under a stream of nitrogen and the trimethylsilyl derivatives were taken up in 0.3 ml hexane.

Paper chromatography was performed on Whatman No. 1 paper impregnated with borate. This paper was prepared by applying a 3 cm band of 0.4 M boric acid to the origin area of the paper and drying before use. The paper was subsequently developed using solvent system 1: Isopropanol-ammonia-water (70:10:20, v/v). Electrophoresis was performed on Whatman No. 43 paper using buffer system 1: water-pyridine-glacial acetic acid (89:1:10, v/v), pH 3.5. Phosphate-containing compounds on paper chromatograms or electrophoretograms were located by spraying according to Hanes and Isherwood (1949).

2.3 Analytical Procedures

Gas chromatography was performed on a Varian 1200 FID gas chromatograph using a 1.8 m x 0.4 cm i.d. stainless steel column packed with 3% methylphenylsilicone on diatomaceous earth. A LKB 9000 gas chromatograph-mass spectrometer combination was also employed using a 10 m x 0.01 cm i.d. glass capillary column coated with methyl silicone. For radioactivity measurements, strips from paper chromatograms

or electrophoretograms were counted in toluene scintillation fluid using a Packard liquid scintillation spectrometer Model 3380..

2.4 Synthetic Procedures

(i) Reduction of DL-Glyceraldehyde with Hydrogen Sulfide. DL-glyceraldehyde (0.1 mmoles) was dissolved in 20 ml water and the resulting solution heated to 85°C under reflux for 16 h. Hydrogen sulfide gas was bubbled continuously through this solution. A control reaction omitting hydrogen sulfide was also performed. On completion of the reaction the water was removed in vacuo at room temperature and the residue dissolved in 5 ml methanol. Aliquots (0.2 ml) were derivatized for gas chromatography and gas chromatography-mass spectrometry as described in Section 2.2.

(ii) Reduction of DL-Glyceraldehyde or Dihydroxyacetone with Hydrazine. DL-glyceraldehyde or dihydroxyacetone (4.49 mmoles) was dissolved in 10 ml water and hydrazine (12.6 mmoles) was added. The solution (pH 8.2) was stirred at 30–31°C and samples (0.1 ml) were withdrawn periodically for derivatization and analysis by gas chromatography and gas chromatograhy-mass spectrometry.

(iii) Reduction of DL-Glyceraldehyde with Formaldehyde. DL-glyceraldehyde (2.23 mmoles) was dissolved in 5 ml water and formaldehyde (4.93 mmoles) and calcium hydroxide (4.59 mmoles) were added. The reaction mixture was stirred at 30–31°C. The initial pH was 12.5, but had dropped to 7.5 by the end of the reaction. Samples (0.1 ml) were withdrawn periodically for derivatization and analysis as previously described.

(iv) Phosphorylation of Glycerol. Aqueous solutions (0.2 ml) containing glycerol (5 μmoles) and ^{32}P-ammonium dihydrogen phosphate (5 or 15 μmole, specific activity 98 μCi/μmole) or ^{14}C-glycerol (5 μmole, specific activity 0.05 μCi/μmole) and ammonium dihydrogen phosphate (15 μmole) were prepared. To these solutions were added urea (50 μmoles) or urea (50 μmoles) and NH_4Cl (50 μmoles) or cyanamide (50 μmoles). A control mixture omitting the condensing agents was also prepared. The solutions were evaporated under nitrogen to dryness and subsequently heated for 16 h at 85°C. On completion of the reaction, the products were dissolved in 1.0 ml 80% ethanol and aliquots of these solutions analyzed by cochromatography or co-electrophoresis with appropriate standards.

2.5 Enzymatic Phosphorylation of Synthetic Glycerol

The putative glycerol (50 nmoles), synthesized by reaction of DL-glyceraldehyde with hydrogen sulfide, was incubated with γ^{32}-P-ATP (8 μmoles), magnesium chloride (8 μmoles), bovine serum albumin (50 μg), β-mercaptoethanol (16 μmoles), Tris-HCl buffer (20 μmoles, pH 8.5), glycerol kinase (5 μg) in a final volume of 0.4 ml for 2 h at 37°C (Chang and Kennedy, 1967). The γ^{32}-P-ATP was prepared by a modification of the method of Schendel and Wells (1973). A control reaction was also performed using authentic glycerol (5 μmoles) and the other ingredients increased by a factor of 1.50. The reaction was terminated by chilling in ice and the mixture immediately applied to a 2 x 8 cm Dowex-1-formate (100–200 mesh) column. The column was eluted with 50 ml water followed by a continuous gradient starting with 300 ml water in the mixing chamber and 300 ml 4 M formic acid in the reservoir. Fractions (8 ml)

were collected at a flow rate of 1.5 ml per min. When aliquots were counted to locate ^{32}P-containing compounds, only a single radioactive peak was detected. The radioactive fractions were evaporated to dryness in vacuo at 10°C, the residue dissolved in 2 ml water, neutralized with ammonia and diluted with 3.5 ml 0.1 M Tris-HCl buffer, pH 5.2. The solution was shaken with activated charcoal (50 mg), centrifuged, and the charcoal washed with the Tris-HCl buffer. The supernatants and washings were neutralized with ammonia and made up to 10 ml with water. Aliquots of these solutions were co-electrophoresed in a buffer system with authentic standards. The electrophoretograms were sprayed to locate P-containing compounds and strip counted to locate radioactive products.

3. Results and Discussion

3.1 Synthesis of Glycerol

Gas chromatographic analysis of the products obtained from the reaction of DL-glyceraldehyde with hydrogen sulfide revealed the presence of a compound with the same retention time as an authentic sample of tris-(trimethylsilyl)-glycerol (Fig. 1, peak a). Other silylated compounds with longer retention times (not shown) were formed and were presumably polymers of glyceraldehyde. The mass spectrum of peak a and that of standard tris-(trimethylsilyl)-glycerol is shown in Fig. 2. Although a molecular ion, m/e 306, was not present, these two spectra were identical except for slight differences in ion intensities. The yield of glycerol was estimated to be 1%. Low yields of glycerol were also obtained when DL-glyceraldehyde or dihydroxyacetone were treated with hydrazine, with product formation reaching a maximum of 3% after a 4 day incubation period. The reduction of glyceraldehyde with formaldehyde also gave approximately 1% yield of glycerol. Although the initial pH of this reaction mixture (pH = 12.5) was higher than generally accepted as prevailing on the primitive Earth, it became essentially neutral (pH = 7.5) at the end of the reaction. This neutralization can also be expected to have occurred on the primitive Earth by the base-catalyzed

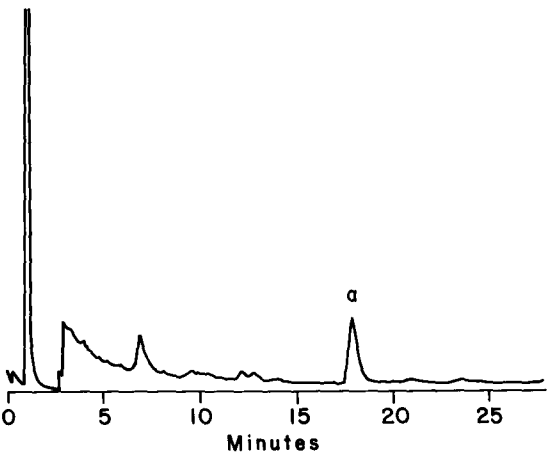

Fig. 1. Gas chromatogram of the products from the reaction of DL-glyceraldehyde with hydrogen sulfide. The product represented by Peak a had the same retention time as authentic tris-(trimethylsilyl)-glycerol

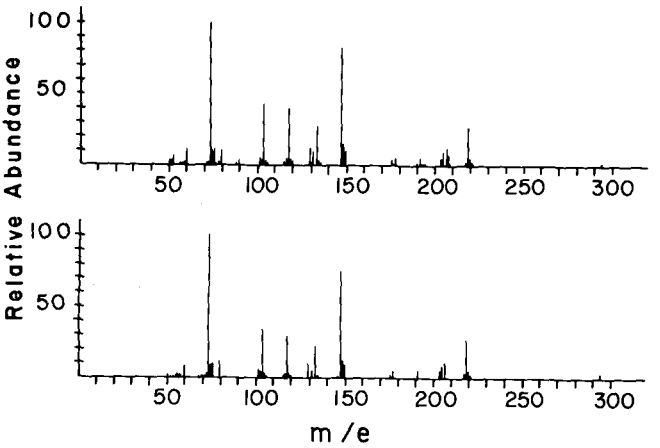

Fig. 2. Mass spectra of synthetic (top) and authentic (bottom) tris-(trimethylsilyl)-glycerol. See text for further details

Cannizzaro disproportionation of aldehydes into acids and alcohols. Control reactions indicated that glycerol was not a contaminant in the DL-glyceraldehyde or dihydroxyacetone used in these experiments.

When the putative glycerol obtained from the reduction of DL-glyceraldehyde with hydrogen sulfide was incubated with γ-^{32}P ATP and glycerol kinase, 75% of the reduction product was converted to a compound which had the same electrophoretic mobility as an authentic sample of glycerophosphate in buffer system 1. Authentic glycerol was converted in 53% yield to glycerophosphate in the enzymatic reaction.

These results show that a variety of reducing agents, namely hydrogen sulfide, hydrazine and formaldehyde, all compounds assumed to have been present on the primitive Earth, can reduce either DL-glyceraldehyde or dihydroxyacetone to glycerol. The yields obtained from these reactions were low, but not unreasonably so for reactions conducted under primitive Earth conditions.

3.2 The Synthesis of Glycerophosphates

The R_f values and electrophoretic mobilities of glycerol-containing standards are shown in Table 1. After heating glycerol and ^{32}P-ammonium dihydrogen phosphate with urea or cyanamide, radioactive compounds which chromatographed on borate-impregnated paper with *sn*-glycero-1(3)-phosphate, glycero-2-phosphate and cyclic glycerophosphate were obtained. The yields of glycerophosphates are given in Table 2. Appreciable formation of glycerophosphates occurred in the presence of either urea or cyanamide and was highest when a three-fold excess of ammonium dihydrogen phosphate was added (reaction series B). The greatest yield of combined glycerophosphates (at least 33%) was obtained with either cyanamide or with a mixture of urea + NH_4Cl. Thus, for example, the addition of the three products of experiment 4B in Table 2 (1.45 μmole 1(3)GP + 0.03 μmole 2GP + 0.39 μmole cGP), is equivalent to a yield of 37.4% not corrected for the loss experienced through recovery. The bulk of the incorporated radioactivity was found in *sn*-glycero-1(3)-phosphate and the remainder was distributed in glycero-2-phosphate and cyclic glycerophosphate. The highest amounts of cyclic glycerophosphate were formed in the presence of cyanamide.

Table 1. Values and electrophoretic mobilities of standard compounds

Compound	Chromatography on borate impregnated paper	Electrophoresis buffer, pH 3.5
sn-glycero-1(3)-phosphate	0.19	0.85
glycero-2-phosphate	0.30	0.85
inorganic phosphate	0.09	1.00
cyclic glycerophosphate	0.72	1.00
glycerol	0.00	0.00

Chromatography and electrophoresis were performed in solvent systems described in the text

Table 2. Synthesis of glycerophosphates from glycerol and phosphate

Reaction series A or B Experiment No.	Glycerol + ^{32}Pi and Addition	Yields of reaction products (in micromolecules)							
		Unreacted Pi		1(3) GP		2 GP		cGP	
		A	B	A	B	A	B	A	B
1.	none	4.98	13.53	0.02	0.14	N.D.	0.01	N.D.	0.09
2.	urea	4.71	11.10	0.07	0.65	0.02	0.35	0.05	0.23
3.	urea + NH_4Cl	4.54	9.90	0.32	1.32	0.05	0.14	0.03	0.19
4.	cyanamide	3.93	9.00	0.50	1.45	0.06	0.03	0.07	0.39
5.	imidazole	4.95	14.21	0.01	0.13	N.D.	0.02	N.D.	0.03
6.	cyanamide + imidazole	4.27	9.32	0.50	1.31	0.07	0.02	0.05	0.35

A: All the experiments (1 to 6) in this reaction series contained 5 μmoles glycerol and 5 μmoles ^{32}P-ammonium dihydrogen phosphate. Recoveries of radioactivity were 91–99%.

B: All the experiments (1 to 6) of this reaction series contained 5 μmoles glycerol and 15 μmoles P-ammonium dihydrogen phosphate. Recoveries of radioactivity were 72–91%.

Other additions were made as indicated in the Table in 50 μmole amounts. All the reaction mixtures were heated for 16 h at 85°C, and the reaction products separated by chromatography on borate-impregnated paper. See text for further details.

Pi = unreacted inorganic phosphate; 1(3)GP = *sn*-glycero-1(3)-phosphate; 2GP = glycero-2-phosphate; cGP = cyclic glycerophosphate; N.D. = Not detected

Almost identical experiments (not shown in Table 2) were performed using ^{14}C-glycerol as radioactive precursor under the conditions of a three-fold excess of non-radioactive inorganic phosphate and the products separated by high voltage electrophoresis. In this case in the presence of cyanamide, 16.6% and 4.7% of the label were found in monoesterified glycerophosphates and cyclic glycerophosphate, respectively, without correction for recovery losses. When urea was added, the corresponding yields were 25.7% and 2.3%.

In summary, these findings indicate that glycerol can be phosphorylated in significant yields (from 21.3% to 37.4%, without counting recovery losses) under evaporating conditions in a reaction dependent on a suitable condensing agent, namely urea, urea + NH_4Cl, or cyanamide. In all cases *sn*-glycero-1(3)-phosphate is the major product formed.

4. Conclusion

In this paper we have shown the synthesis of both glycerol and *sn*-glycero-1(3)-phosphate under conditions which may have occurred on the primitive Earth. These results are significant when considered in conjunction with our earlier work on the synthesis of acylglycerols (Eichberg et al., 1977) and phosphatidic acids (Epps et al., 1978). The formation of lipids, particularly phospholipids, would have been of fundamental importance in the evolution of lipid membranes and the self-assembly of the first cellular organism (Oró et al., 1978).

Acknowledgments. This work was supported in part by NIH grant NS12493 and grant E-675 from the Robert A. Welch Foundation to J. Eichberg and by NASA grant NGR 44–005–002 to J. Oró.

References

Chang, Y.Y., Kennedy, E.P. (1967). J. Lipid Res. **8**, 447–455
Eichberg, J., Sherwood, E., Epps, D.E., Oró, J. (1977). J. Mol. Evol. **10**, 221–230
Epps, D.E., Sherwood, E., Eichberg, J., Oró, J. (1978). J. Mol. Evol. **11**, 279–292
Hanes, C.S., Isherwood, F.A. (1949). Nature **164**, 1107–1112
Lapidot, Y., Barzilay, I., Hadju, J. (1969). Chem. Phys. Lipids **3**, 125–134
Lohrmann, R., Orgel, L.E. (1971). Science **171**, 490–494
Oró, J., Sherwood, E., Eichberg, J., Epps, D.E. (1978). In: Light transducing membranes (D. Deamer, ed.) pp. 1–19, New York, Academic Press
Schendel, P.F., Wells, R.D. (1973). J. Biol. Chem. **248**, 8319–8321
Schwartz, A.W. (1972). Biochem. Biophys. Acta **281**, 477–480

Received May 25, 1979; Revised August 20, 1979