Origins of Life and Evolution of Biospheres (2005) 35: 323–332

THE FORMATION OF GLYCEROL MONODECANOATE BY A DEHYDRATION/CONDENSATION REACTION: INCREASING THE CHEMICAL COMPLEXITY OF AMPHIPHILES ON THE EARLY EARTH

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(Received 3 April 2004; accepted in revised form 12 July 2004)

Abstract. Dehydration/condensation reactions between organic molecules in the prebiotic environment increased the inventory and complexity of organic compounds available for self-assembly into primitive cellular organisms. As a model of such reactions and to demonstrate this principle, we have investigated the esterification reaction between glycerol and decanoic acid that forms glycerol monodecanoate (GMD). This amphiphile enhances robustness of self-assembled membranous structures of carboxylic acids to the potentially disruptive effects of pH, divalent cation binding and osmotic stress. Experimental variables included temperature, water activity and hydrolysis of the resulting ester product, providing insights into the environmental conditions that would favor the formation and stability of this more evolved amphiphile. At temperatures exceeding 50 °C, the ester product formed even in the presence of bulk water, suggesting that the reaction occurs at the liquid interface of the two reactants and that the products segregate in the two immiscible layers, thereby reducing hydrolytic back reactions. This implies that esterification reactions were likely to be common in the prebiotic environment as reactants underwent cycles of wetting and drying on rare early landmasses at elevated temperatures.

Keywords: amphiphiles, ester bond, membranes, prebiotic, vesicles

Introduction

A membranous boundary structure is essential for the origin of cellular life. Laboratory simulations of prebiotic membranes have been explored (Hargreaves *et al*., 1977; Walde *et al*., 1994; Apel *et al*., 2002; Hanczyc *et al*., 2003), but relatively few attempts have been made to determine whether such membranes could exist in the marine environment generally considered to be the site of life's origin. Carboxylic acids as short as 8 carbons have been shown to self-assemble into membranous vesicles capable of encapsulating and protecting functional macromolecules (Apel *et al*., 2002). However, these membranes are unstable in the presence of divalent cations, acidic pH ranges and high ionic strength that would characterize an early ocean (Knauth, 2002). For this reason we are investigating the possibility that

simple condensation reactions may have provided more complex molecules that can assemble into stable membranes in a marine environment.

All scenarios for prebiotic evolution leading to the origin of life assume that non-enzymatic condensation reactions were responsible for polymer synthesis such as peptide bond formation between amino acids, and phosphodiester bonds between nucleotides. The resulting polymers would presumably have increasingly complex emergent properties. It would be desirable to have a relatively simple model system that will permit analysis of a dehydration/condensation reaction, and to this end we have chosen ester formation between alcohols and monocarboxylic acids (Hargreaves *et al*., 1977). The resulting products form relatively stable bilayer membranes that can withstand disruptive effects of high ionic strength and the presence of divalent cations (Monnard *et al*., 2002). Figure 1 clearly shows the presence of stable vesicles of GMD/decanoic acid in solution with NaCl concentration above that which destabilize and destroy vesicles of decanoic acid alone. These monoglycerides, although abiotically produced, are one step closer to the lipids common in today's cells (selected by Darwinian evolution) and would have facilitated the colonization of the marine environments by primitive cellular life.

Here we report a thermodynamic and kinetic analysis of a model esterification reaction that addressed the following questions:

1. What temperature ranges are necessary to provide sufficient activation energy to permit esterification?

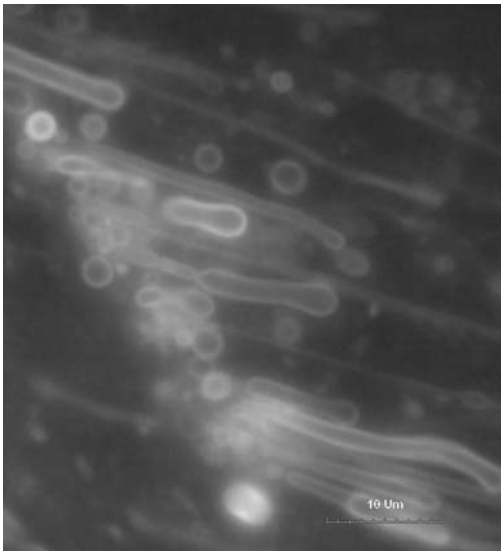

Figure 1. Vesicles of GMD (7 mM)/decanoic acid (14 mM) are clearly visible in a solution of 466 mM NaCl at pH 7.3. At this concentration of NaCl, vesicles of decanoic acid alone are completely disrupted.

2. What is the minimum relative humidity that promotes esterification while still inhibiting the back reaction of hydrolysis?
3. Can the products form stable membranes in the presence of divalent cations over a range of pH?

Materials and Methods

Carboxylic esters can be prepared by heating carboxylic acids with an alcohol, a reaction that is classified as a Fischer esterification.

$$\mathrm{RCOOH} + \mathrm{R'OH} \Leftrightarrow \mathrm{RCOOR'} + \mathrm{H_2O}$$

Fischer esterification reactions are reversible, so the yield of esters is limited by the equilibrium constant K_e.

$$K_e = \frac{[\text{ester}][\text{water}]}{[\text{acid}][\text{alcohol}]}$$

Equilibrium constants for esterification using alcohols and sterically unhindered acids normally fall in the range of $K_e = 1$–10. The K_e for the reaction of acetic acid with ethanol has been experimentally determined to be 3.8 (Palleros, 2000).

If this reaction is initiated with one mole of each reactant and a is the mole fraction of product:

$$K_e = \frac{[a][a]}{[1-a][1-a]} = \frac{a^2}{(1-a)^2} = 3.8$$

Then $a = 0.66$, representing a 66% yield.

The yield may be increased by using an excess of one of the reactants, or by removing one of the products from the reaction mixture. The reverse reaction, or hydrolysis, can be minimized by removing *either* product from the reaction mixture. This application of Le Chatelier's principle is relevant to our experimental results and will be discussed in more detail later.

Our experimental approach focused on the production of glycerol monodecanoate (GMD), an amphiphile that is effective in reducing the sensitivity of decanoic acid (DA) vesicles to the disruptive effects of NaCl and divalent cations. The reaction then becomes:

$$\mathrm{DA} + \text{glycerol} \Leftrightarrow \mathrm{GMD} + \text{water}$$

The reaction proceeds by nucleophilic attack by the oxygens of the alcohol on the acid-bearing carbons of the carboxylates to form an ester bond. If monodecanoate

is a product, the ester can be on either the 1- or 2- carbon of the glycerol. If the ester forms in the 1- position, then carbon 2- on the glycerol becomes a steric center. Therefore there are 3 monodecanoate products possible. If a di-decanoate is formed the ester linkages may form on the 1- and 2- carbons or the 1- and 3- carbons of the glycerol. In the first case carbon 2- remains a steric center. In the second case there is no steric center. These constitute three more possible products. The final case is the formation of a tri-decanoate. In total there are seven products possible from this reaction. We limited our study to the formation of monoglyceride, but it should be noted that small amounts of the di- (GDD) and triglyceride (GTD) were also detected.

Temperature dependence of the esterification reaction

Equal amounts (1.4×10^{-4} moles) of melted decanoic acid (24.5 μL) and glycerol (20 μL of 50% glycerol in water) were deposited in borosilicate test tubes (85×100 mm) and heated in air to specified temperatures for a period of three days. Under these conditions the water originally present was quickly lost by evaporation. Each day one tube was removed from the oven and placed in a vacuum desiccator at room temperature until all three tubes were removed. The reaction was repeated at temperature intervals of 10 °C between 50 °C and 110 °C.

At the end of a three day reaction cycle, each set of reaction tubes was acidified (25 μL of 37% HCl), and extracted with 2 mL of chloroform:methanol solution (2:1) and 1 mL of water. The solvent was mixed on a vortex stirrer and the immiscible layers were allowed to separate. The top layer of water/methanol containing the unreacted glycerol was removed by vacuum aspiration. The bottom layer of chloroform, which contained the unreacted, protonated decanoic acid and the ester products, was spotted on TLC plates (Whatman, 250 micrometer coating of silica gel, 10 cm $\times$ 10 cm). Aliquots (50 μL) from each of the three tubes were dried as $\sim$5 mM spots on each TLC plate and a fourth row was spotted with 50 μL of GMD standard solution (10 mg per mL of chloroform). The one-dimensional plates were run with a hexane:ethyl ether:acetic acid solution (100:200:1) followed by exposure to iodine vapor for 15 min to stain the separated fractions. The remaining solution was placed in a vacuum dessicator overnight at room temperature to remove the chloroform. The presence of ester product was confirmed using TLC and later measured quantitatively as described below.

Measurement of ester bond formation

To measure the yield of the ester product, a variation of the ferric hydroximate test was employed (Palleros, 2000). Each residue from the chloroform:methanol:acetic acid extraction was dissolved in 1 mL of hydroxylamine HCl solution (0.5 M in 95% ethanol) and vortexed until the pellet dissolved. 0.4 mL of 6 M NaOH was added to the resulting solution, which was stirred by a vortex mixer, and warmed

in a heating block for 5 minutes at 110 °C. Under these conditions NH_2OH reacted stoicheometrically with each ester bond to form one molecule of hydroxamic acid and glycerol. Each solution was acidified with 2.5 mL of 1 M HCl, followed by addition of 2 mL 95% ethanol to clarify the turbid mixture. Ferric chloride solution (50 μL, 5% by weight) was then added to each tube and again stirred on a vortex mixer. Hydroxamic acid coordinates to ferric ions in bidentate octahedral symmetry to form a highly colored complex (Huheey *et al.*, 1993). Absorbance at 490 nm was used to determine ester synthesis by comparison with standard curves prepared with known amounts of GMD.

Results

Chromatographic analysis of dehydration/condensation products

Reactions between decanoic acid and glycerol were carried out at temperatures ranging from 50–110 °C. Products were extracted and analyzed first on TLC plates. Exposure to iodine vapor revealed the GMD product, the unreacted decanoic acid, and traces of glycerol didecanoate and tridecanoate (Figure 2). The R_f value for the large lower spot in lane #1 is the same as the GMD standard in lane #2 (0.17). The R_f values for glycerol di-decanoate (GDD) and glycerol tri-decanoate (GTD) were 0.6 and 0.67, respectively. The R_f value of the unreacted decanoic acid matched that of the decanoic acid standard as well (0.87). No GMD product was detected when the reaction was run at temperatures lower than 50 °C.

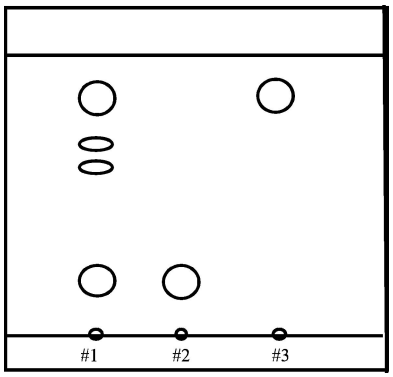

Figure 2. TLC analysis. One-dimensional TLC plate developed in iodine vapor. Small circles on lower line indicate initial positions of samples. Lane #3 was a standard spotted with decanoic acid in chloroform. Lane #2 was a standard spotted with racemic GMD (Sigma). Lane #1 shows typical products of the reaction: Large lower spot GMD and two ovoid spots indicating the minor products GDD and DTD. The upper spot in lane #1 is the unreacted decanoic acid.

QUANTITATIVE RESULTS OF DEHYDRATION/CONDENSATION REACTION

The products of the experiment were extracted and analyzed by the ferric hydroximate test for esters. As expected, the yield of ester was temperature dependent (Figure 3) with more ester produced at higher temperatures. Figure 4 shows the production of GMD expressed in percent of theoretical yield versus temperature. The highest yield over a three day period was 35% at 90 °C. Subsequent experiments focusing on hydrolysis of GMD were therefore carried out at this temperature.

We calculated the equilibrium constant K_e at 90 °C using the following data:

$$K_e = \frac{[0.35]^2}{[1-0.35]^2} = 0.28$$

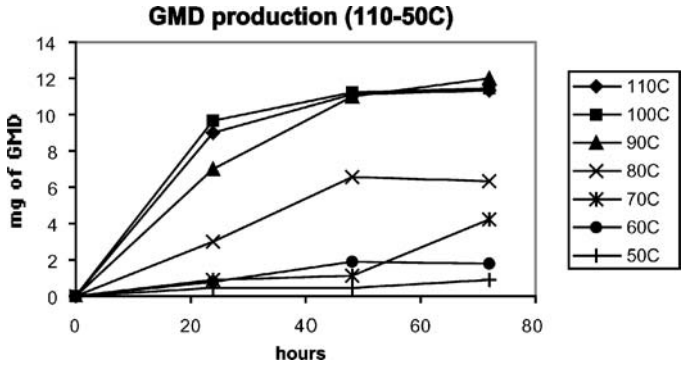

Figure 3. GMD production vs. time. The graph above shows mgs of GMD produced over the course of 3 days at temperatures ranging from 50–110 °C. No ester products were formed at temperatures below 50 °C. Equal molar amounts (1.4×10^{-4} moles) reactants (decanoic acid and glycerol) were used in each trial.

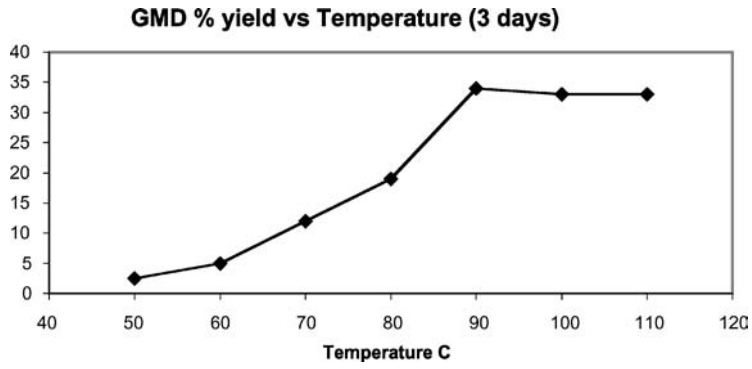

Figure 4. GMD % yield vs. temperature. The above graph shows the amount of product formed in the esterification reaction given as a % of the theoretical yield after a period of three days in the oven.

The calculation assumes that water molecules produced by esterification remain in a 1:1 mole ratio with the product GMD presumably dissolved in the glycerol phase. Because concentrations were difficult to estimate under our experimental conditions in which immiscible fractions of bulk phase fatty acid and glycerol were the reactants, it was necessary to use percent yield rather than actual concentrations in the equation. The resulting K_e value is significantly lower than the literature value for the esterification reaction between acetic acid and ethanol ($K_e = 3.8$) described earlier (Palleros, 2000).

GMD ESTER SYNTHESIS IN THE PRESENCE OF WATER

At the time of the origin of life, a plausible senario is that the Earth was covered by a relatively shallow ocean in which volcanic landmasses occasionally appeared (Knauth, 2002). The interface between the atmosphere, lava mineral surfaces and sea water would provide a fluctuating environment in which condensation reactions could be driven by heat combined with wet/dry cycles. If we assume that ester formation was necessary to provide stable membrane structures for primitive life in marine environments, condensation reactions similar to that which forms GMD were likely to be involved. The model system we have described here permitted us to ask what relative humidity was required to facilitate such reactions, and whether the presence of liquid water or high humidity would impede amphiphilic ester synthesis. Fischer esterification reactions between a carboxylic acid and alcohol are reversible unless the water product is removed to drive the forward reaction. An experiment was therefore designed to monitor GMD production in the presence of known amounts of water.

The procedure described in Methods was employed with modifications. Instead of glycerol in water, mixtures of glycerol in methanol (50%) were placed in 3.5 mL glass ampoules and the methanol evaporated in a 90 °C oven overnight leaving 1.4×10^{-4} moles of glycerol in each tube. An equal molar amount of decanoic acid was then added to the tubes together with increasing amounts of liquid water. The ampoules were then sealed and placed in an oven at 90 °C for three days, then opened and the products quantified by the ferric hydroximate test for esters.

The first ampoule contained no water and the expected amount of GMD was formed (Figure 5). Note that there was no difference detected in the amount of GMD product formed in the presence of 5 μL of water. This is twice the number of moles of water that would have been formed had the reaction proceeded to the theoretical yield. Ten times this amount of water (50 μL) did little to impede the formation of GMD. Only in the presence of 100 μL of water, or twenty times the molar amounts of both reactants, was the reaction entirely inhibited. To better understand this result, the hydrolysis of GMD esters was examined in closer detail.

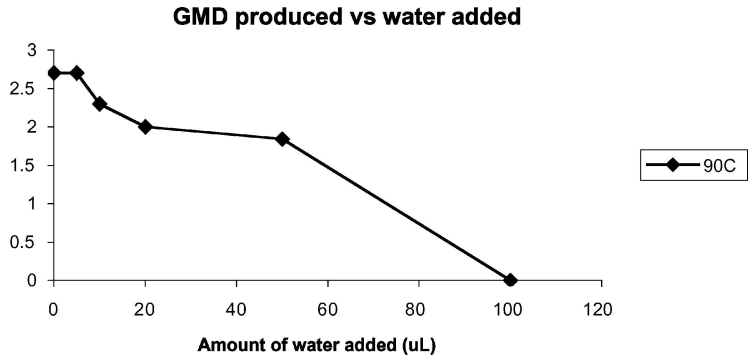

Figure 5. GMD produced (mg) in 3 days versus increasing amounts of water added. Equal molar amounts of the two reactants (1.4×10^{-4} moles) were sealed in 3.5 mL ampoules with varying amounts of water added before sealing.

HYDROLYSIS OF GMD ESTER

An experiment was designed to measure hydrolysis of GMD, the reverse of Fischer esterification. Ten mgs of GMD (Sigma) were placed in each of 3.5 mL sealable ampoules. One series was allowed to stand at room temperature for three days while the other series was incubated for the same amount of time at 90 °C. One ampoule in each series contained no water. To the remaining containers were added increasing amounts of water up to 200 μL/ampoule. Each ampoule was then sealed and after 72 h the containers were opened and extracted. The remaining GMD was quantified as before by ferric hydroximate test for esters.

At the end of three days at 90 °C, 60% of the original GMD remained even in the presence of 200 μL of water (Figure 6). Only slightly more (70%) remained for the corresponding ampoule (200 μL water) incubated for the same time period at room temperature. The hydrolysis reaction, like the ester forming condensation reaction, did not seem to be directly related to the amount of water present. However,

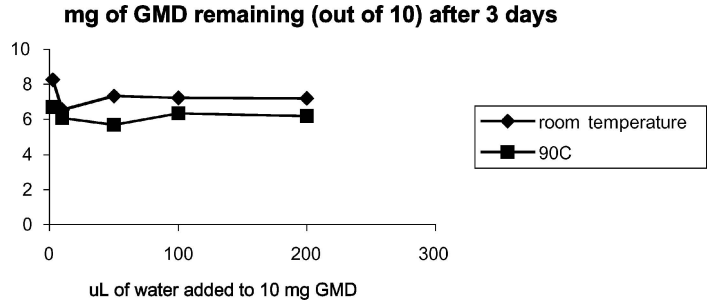

Figure 6. Hydrolysis of GMD. Starting with 10 mgs of GMD, this figure compares the amount of GMD remaining after three days of incubation with varying amounts of water at two temperatures.

unlike the forward reaction, the hydrolysis of GMD revealed less dependence on temperature to proceed. Given that GMD synthesis could have taken place in a slightly acidic volcanic setting, it would be of great interest to investigate the pH dependency of the hydrolysis reaction.

Discussion

The results reported here show that temperature is the most significant factor in driving a Fischer esterification reaction that produces an amphiphilic product. Higher temperature ranges provided activation energy and reduced the relative humidity of the reaction conditions, so that there was a strong temperature dependence of the condensation reaction between decanoic acid and glycerol. The highest yield of the ester product was 35% of the theoretical yield at 90 °C and no GMD was produced at temperatures less than 50 °C.

At the beginning of this study, we expected to find a greater sensitivity to the presence of water, so that the reaction would be controlled by the relative humidity of the reaction chamber. Instead we found that bulk water in the reaction only affected the yield or the rate of hydrolysis when present in considerable excess. The reaction was heat driven, and the GMD product much more stable and resistant to hydrolysis than expected. Figure 7 suggests one reason for this. If 1 mL of decanoic acid is placed in a test tube with 1 mL of glycerol at 60 °C, the two liquids are clearly immiscible. The esterification reaction therefore may be largely confined to the interface between the two liquids, with products dissolving either

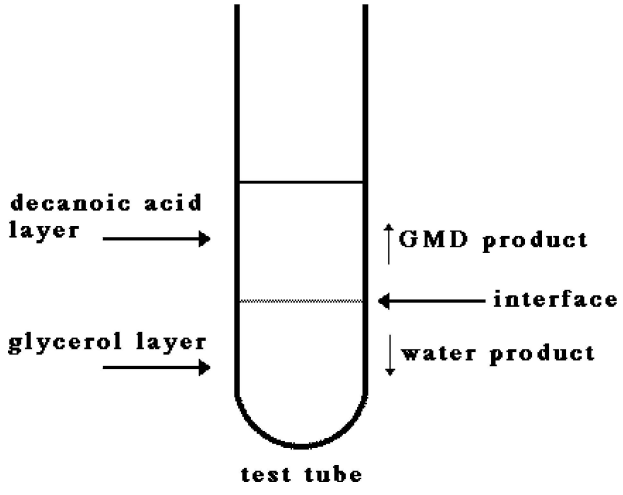

Figure 7. Diagram of the reaction forming GMD. The two reactants are immiscible interacting only at the interface between layers. The two products are soluble in different layers and are thus segregated, minimizing the reverse hydrolysis reaction.

in the glycerol phase or in the decanoic acid phase. The fact that GMD esters can form in the presence of an excess of water and that the hydrolysis reaction does not seem to depend on the concentration of water may be explained using the same logic. That is, as products are synthesized at the interface, they segregate into the two phases so that the reverse hydrolysis reaction becomes less probable.

We conclude that a plausible prebiotic environment for ester synthesis would be sites having sustained temperatures over 50 °C. Potential energy sources include solar heating of volcanic mineral surfaces and geothermal environments resembling the land-air-water interfaces of Hawaii. Future experiments are planned in which solar and geothermally heated mineral surfaces will be used as substrates for esterification reactions.

Acknowledgments

The authors wish to thank Pierre-Alain Monnard for the photo of GMD vesicles in high salt from his extensive archive. This work is supported by NASA grants NAG5-4665 and SC-00-35.

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