Racemization of amino acids under natural conditions: part 3—condensation to form oligopeptides

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The condensation reaction peptide$_n$ + amino acid → peptide$_{n+1}$ in water is thermodynamically unfavourable by about 2.3 kcal/mol per peptide bond at ~ 30°C and near neutral pH for glycine, due to the stability of zwitterions. An equilibrium ratio of [peptide$_{n+1}$]/[peptide$_n$] of ~ $^1/_{50}$ results. For [alanyl-glycine]/[alanine + glycine] the proportion is only about 10^{-5} under those conditions. Accelerated peptide growth by reactions such as 2 peptide$_n$ ⟶ peptide$_{2n}$ can only occur in highly concentrated laboratory conditions. Chemically esterifying end carboxyl groups to activate them, sonication, ideal stoichiometries, pH, temperature and pressure are examples of intelligent intervention to force the desired outcome.

Examining reaction parameters systematically (initial concentration of glycine, dehydration times, number of dehydration cycles, temperature, pH, and concentration of NaCl) produced as largest peptide Gly_{12} in aqueous solution and Gly_{14} embedded in an insoluble solid. Mass spectrometry analysis suggested Gly_{20} formed in trace amounts. Products had to be quickly removed after being formed to prevent chemical degradation since amino acids are irreversibly destroyed at ~ 240°C. These experiments demonstrate peptides of relevant size for origin-of-life speculations cannot have arisen naturalistically.

Conditions for peptide condensation reactions

Living organisms depend on proteins to carry out a multitude of functions. These must be composed of L-enantiomer amino acid residues (L-peptide$_n$) to produce reliable secondary structures such as α-helices, β-sheets, and turns which are necessary for stable folding into tertiary structures. Enantiopure amino acids (AAs) will racemize, whether in free form or chemically bound, as we discussed in part 2 of this series.[1] Much effort has been expended by the pro-evolution research community on a second problem—namely, to find naturalistic conditions to form long peptides. We will review some key reports on this topic, considering their plausibility for origin of life (OoL) purposes.

L-peptide$_n$ must be large and homochiral and both requirements must be satisfied concurrently if an OoL experiment is to have any significance. In part 4 of this series, we will show that optimizing the conditions to obtain larger peptides inevitably racemizes the peptides faster. This fact is hidden by using glycine in the publications, the only proteinogenic AA lacking D and L enantiomers, and thereby incapable of producing folded proteins.

In part 4 we will compare kinetic rate constants and Gibbs free energy changes for racemization and condensation in water. To facilitate this analysis, let us review peptide condensation.

Naturalistic models to drive amino acid condensation

We will not attempt here a detailed review of all the efforts to overcome the unfavourable thermodynamics and kinetics to form polypeptides in aqueous solution under allegedly plausible naturalistic conditions. Without the use of specialized catalysts, very low yields (typically < 1%) of oligomeric products having $n > 3$ residues have been reported when attempting peptide synthesis on clays[2], minerals[3], at air–water interfaces,[4] on metal oxide surfaces,[5] and under hydrothermal conditions.[6,7] Inevitably only glycine was used, intelligently organized to optimize the intended outcome with no relevance to how unguided processes function in nature. We decided not to analyze those studies which are not realistically amenable to any abiogenesis model.

The overall free energy change of hydrolyzation is unfavourable

For n residues linked in a peptide, there are $n - 1$ peptide bonds. The free energy of peptide bond hydrolysis vs formation in aqueous solution strongly favours the dissociated, non-condensed products. This is because at pH around 4.5 < pH < 7.5, AAs and peptide fragments form ions which are very stable in water. Therefore, a high free energy is required to generate the neutral form of the reactants necessary to undergo condensation reactions.

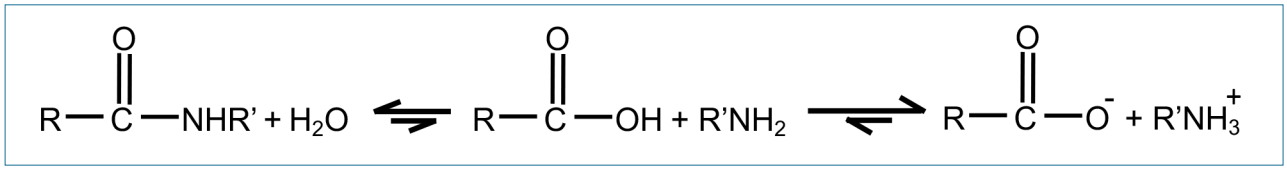

Figure 1. Peptide bond hydrolysis resolved into two contributions: 1) amide bond hydrolysis to neutral carboxylic acid and amino groups, and 2) ionization of these two groups. For amino acids, the charged carboxyl and amino groups are on the same molecules, called 'zwitterions'. (Based on a figure in ref. 8.)

To study equilibrium-rate constants and Gibbs free energy, one can begin from either side of the reaction (figure 1). The reverse of condensation, hydrolysis of a peptide bond to neutral products, is unfavourable energetically, *contra* what most believe; but when it occurs, both fragments generated can then be transformed to the stable aqueous ions.[8] This is what makes hydrolysis favourable overall (figure 1). This insight is symmetrical. The main reason that the formation of AA condensation products in water is unfavourable thermodynamically is the stability of the initial zwitterions in water.

The overall free energy change of hydrolyzation, ΔG_h, thus includes two effects:

$$\Delta G_h = \Delta G_m + \Delta G_i \qquad [1],$$

Table 1. Peptide bond hydrolysis free energy[a] and equilibrium constant for glycine (G) in water, 4.5 < pH < 7.5, 25–37°C[8]

Reaction	$-\Delta G_i$	$-\Delta G_m$	$-\Delta G_h$	K_h, M[c]
GG → G + G	9.9	6.3	3.6[b]	373 ≈ 400
GGG → G + GG	7.8	5.3	2.5	61 ≈ 50
GGG → GG + G	8.8	6.3	2.5	61 ≈ 50
GGGG → G + GGG	7.6	5.3	2.3	44 ≈ 50
GGGG → GG + GG	6.7	5.3	1.4	10
GGGG → GGG + G	8.7	6.3	2.4	52 ≈ 50
polyG → G + $polyG_{-1}$	7.6	5.3	2.3	44 ≈ 50
polyG → $polyG_{-1}$ + G	8.7	6.3	2.4	52 ≈ 50

[a] Free energy changes (ΔG) in kcal/mole at 25°C or 37°C. The change in temperature does not affect the values within their uncertainties of about 0.2 kcal/mole. Reference state is 1 M.

ΔG_h is the overall free energy of amide hydrolysis, including the ionization step.

ΔG_m is the free energy of hydrolysis of the amide bond to uncharged products.

ΔG_i is the free energy of ionization.

[b] Experimental value. All other values in the last two columns were calculated by the author.

[c] Using the relationship $-\Delta G_h = 2.3RT \times \log K_h \approx 1.4 \log K_h$ kcal/mole.

where ΔG_m is due to hydrolysis of the amide bond to uncharged products and ΔG_i is the free energy of their ionization.[8]

Martin from the University of Virginia calculated the overall ΔG_h of hydrolysis for glycine (G),[8] such as via GG ⇌ G + G; GGGG ⇌ GG + GG; and poly-G_n ⇌ poly-G_{n-1} + G (table 1).[8] G + G ⇌ GG was energetically least favourable with an equilibrium constant $K_h \approx {}^1/_{400}$; for $polyG_{n-1}$ + G ⇌ $polyG_n$, $K_h \approx {}^1/_{50}$; and GG + GG ⇌ GGGG $K_h \approx {}^1/_{10}$. (The values in the last column of table 1 were rounded off, as the authors of ref. 8 also did.)

At equilibrium in aqueous solution, ambient temperatures, and a reference concentration of [G] = 1 M, [$polyG_n$] / [$polyG_{n-1}$] will form at a decreasing proportion of ~ $^1/_{50}$ per residue added

$$G \underset{400:1}{\overset{G}{\rightleftharpoons}} GG \underset{50:1}{\overset{G}{\rightleftharpoons}} GGG \underset{50:1}{\overset{G}{\rightleftharpoons}} GGGG \underset{50:1}{\overset{G}{\rightleftharpoons}} \cdots \qquad [2]$$

Therefore, the proportion of $polyG_n$ to G for *n* residues at equilibrium is approximately

$$[polyG_n] / [G] = {}^1/_{400} \times ({}^1/_{50})^{n-2} \qquad [3]$$

A small $n = 101$ residue Gly_{101} would only be present in a molar ratio to free glycine of $< 10^{-170}$ at equilibrium at ~ 25–37°C ($^1/_{400} \times (^1/_{50})^{99}$). The general conclusions from table 1 should apply to other AAs. Hydrolysis should be even more favourable due to steric interference for non-Gly residues. Conversely, condensation will be more difficult for AAs containing a bulkier side chain *R* than H, as found in glycine. Thaxton, Bradley, and Olsen calculated an average free energy of – 3.0 kcal/mol per bond.[9]

Having started with only monomeric G, GG must form before GGG can be produced, and so on for larger peptides. Since the hydrolysis reaction $polyG_n \rightarrow polyG_{n-1}$ + G is always strongly favoured, and the peptides would be in extremely low concentrations in a plausible abiotic environment, a prohibitively long amount of time would be needed to form condensation product $polyG_{n+1}$, which, even at equilibrium, would only have a concentration ~ $^1/_{50}$[$polyG_n$]. Therefore, we neglected possibilities like reaction [4], which would require two molecules both of extreme low concentration to encounter each other and condense, even

though thermodynamically less prohibitive than G + GG → GGG.

$$2\ GG \underset{10:1}{\rightleftharpoons} GGGG \qquad [4]$$

Bada estimated the concentration of amino acids in a putative abiotic ocean to be ~ 10^{-10} M,[10] so at equilibrium a dimer would only have been ~ $^{1}/_{400}$ x 10^{-10} M. Two dimers would therefore be exceedingly unlikely to encounter each other to form GGGG.

Although true that the free energy necessary to condense two peptides is not as high as for two free amino acids, they would be too dilute for this to have been the primary path to form long peptide chains. For abiogenesis purposes, a large environment such as an open ocean is needed to provide the quantity of AAs necessary for a reasonable number of larger peptides to be generated. However, any small peptide would diffuse and be present in very dilute concentrations, so we conclude that k[GG][G] > k'[GG][GG], especially for peptides larger than GG.

Brack reported even less attractive numbers for condensations of other AAs. The free energy for the condensation of alanine and glycine to form alanyl-glycine in H_2O was calculated to be + 4.13 kcal/mol at 37°C and pH 7. The equilibrium concentration of the dipeptide for 1 M solutions of the free AA is only slightly above 10^{-5} M.[11]

Germane to part 4, immense amounts of time would be needed to build up peptides naturally and during the entire time the amino acids would be racemizing.

Oligomerization under high temperature and pressure

Since larger peptides don't form in water naturally, considerable effort has been dedicated to finding suitable conditions to make this possible. Matsuno, at the Nagaoka University of Technology, designed a flow reactor intended to simulate a submarine hydrothermal environment, although he acknowledges some large differences; for instance, in pH, CO_2, Na, and Cl contents.[12] In one series of experiments, 100 mM solutions of glycine dissolved in pure water were introduced into a high-temperature chamber set at 24.0 MPa pressure, which is slightly above the pressure of the critical point of water, thus keeping it liquefied. The effect of temperature was examined between 110° and 350°C in different runs, with 200°–250°C giving the best condensation results.

After a few seconds in the hot chamber, liquid was forced out into a cold chamber containing water also at 24.0 MPa near 4°C to prevent dissociation reactions such as decarboxylation, deamination, and dehydration. The fluid flowing out of the low-temperature chamber was first depressurized to normal atmospheric pressure, and 5 µl aliquots were examined with HPLC. High pressure was reapplied to force the flow back into the original hot chamber. In this manner solutions producing oligopeptides were repeatedly reintroduced as reactants, using very short cycle times of 34 and 78 s. Outside such a laboratory setup any oligopeptides formed would diffuse into the huge ocean volume instead, remaining in place and permitting further condensation. After about ten minutes the yields no longer increased for the desired products: diketopiperazine (a cyclic peptide formed by dimerizing two glycines) (> 1% yield), the glycine dimer (~ 1%) and glycine trimer (< 1%). No larger peptides were found. The absence of tetraglycine suggests that it was rapidly hydrolyzed into two molecules of diglycine. These were decidedly unspectacular results.

In a second set of experiments, 10 mM $CuCl_2$ was added to the 100 mM glycine solution at a pH of 2.5 (to facilitate condensation), at 250°C and 24.0 MPa in the hot chamber. The cycle time was maintained at 34 s. Now four different oligomers were found, with the following yields after 30 minutes: diketopiperazine (~ 0.1%), $(Gly)_2$ (< 0.01%), $(Gly)_4$ (~ 0.1%), and $(Gly)_6$ (~ 0.001%).

The copper ions seem to have prevented the hydrolysis of tetraglycine. The authors interpreted the presence of even-numbered oligopeptides up to hexaglycine and the absence of both tri- and pentaglycine to mean that the chain elongation proceeds mainly by aminolysis of diketopiperazine.

The experiments were cleverly terminated after such a short time to prevent degradation of the products. The results confirm the work conducted by Bada in 1995 to evaluate hydrothermal vent type chemistry, who concluded that amino acids are irreversibly destroyed by heating at ~ 240°C.[13]

In later work, reported in 2000, Matsuno *et al.* worked with 40 mL L-alanine, which has no reactive side groups, at 250°C and identified $(Ala)_2$ (< 1%), $(Ala)_3$ (< 0.01%) and $(Ala)_4$ (~ 0.001%). In the same paper, they optimized the conditions and re-examined 100 mM glycine mixed with 10 mM $CuCl_2$ for a longer experimental time (2 hrs) and managed to produce $(Gly)_8$ in ~ 0.001% yield.[14] The low yield reflects decomposition due to longer exposure to high temperature.[14]

These experiments demonstrate that hydrothermal vent-type environments are not plausible environments for OoL models. The carefully optimized experimental conditions included high concentrations of initial AA at a boundary between ~ 250°C and icy water; recirculation of peptides at an optimal rate to concentrate them in a high-pressure, low-volume region against a high temperature and pressure gradient. Even then it was only possible to generate $(Ala)_4$ and $(Gly)_8$ at concentrations of ~ 0.001% after about two hours (after which they rapidly decomposed thermally).

But this work is valuable for our purposes. It illustrates a point we will emphasize in part 4. The environmental parameters modified to accelerate condensation will also

accelerate racemization. In this case, a large amount of Cu^{2+} was indispensable to obtain some larger oligomers, but the cation would also accelerate the rate of AA racemization dramatically.[1,15] In addition, we want to compare racemization and condensation rates at all temperatures, and Matsuno *et al.* have given us important empirical data for how long it would take to form small peptides at the high temperature of 250°C in water under pressure. We will use the Arrhenius equation in part 4 to show that L → D conversion under these conditions would be several orders of magnitude faster than condensation.

Table 2. Oligomer concentrations as a function of number of hydration–dehydration cycles at 130°C after 24 h. Yields calculated as a percentage of the glycine (Gly) starting material.[21]

Oligomer	Cycle 1	Cycle 2	Cycle 3	Cycle 4
$(Gly)_2$	13.96	10.26	9.42	8.36
$(Gly)_3$	10.4	7.7	6.46	5.41
$(Gly)_4$	7.61	5.95	5.11	4.41
$(Gly)_5$	5.11	4.23	3.53	3.07
$(Gly)_6$	3.64	3.71	3.37	3.03
$(Gly)_7$	1.91	2.07	1.94	1.67
$(Gly)_8$	1.91	2.05	1.07	0.64
$(Gly)_9$	1.09	1.3	0.77	0.66
$(Gly)_{10}$	0.81	0.93	0.81	0.74
$(Gly)_{11}$	0.2	0.32	0.28	0.26
$(Gly)_{12}$	0.4	0.56	0.4	0.38
$(Gly)_{13}$	0.11	0.34	0.3	0.25

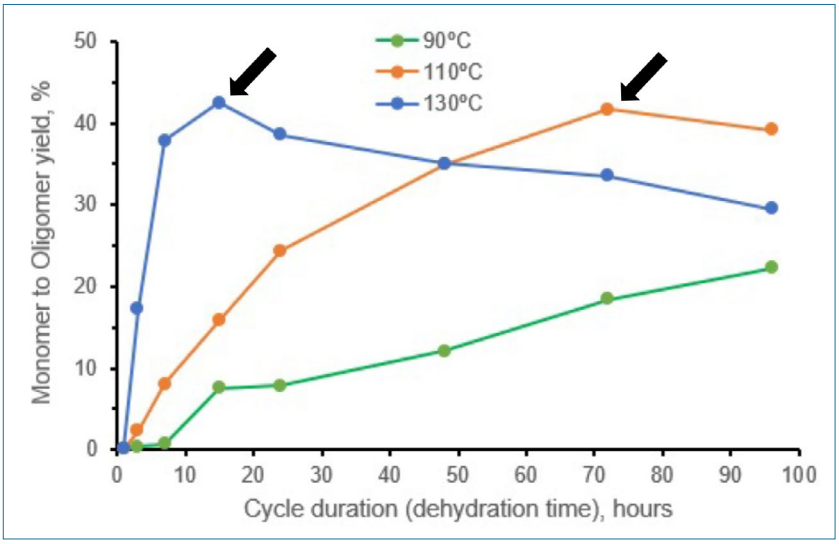

Figure 2. Oligomer yield at different temperatures as a function of experimental cycle duration (i.e. dehydration time).[20]

Scenarios to minimize hydrolysis of peptides

Special environmental conditions have been proposed to counteract the unfavourable AA condensation thermodynamic and kinetic effects. One environment involves thin hydrophobic air–water interfaces such as those potentially found at the surfaces of lakes and oceans, and in atmospheric aerosols.[16] Griffith and Vaida conducted some experiments using a Langmuir-trough and chemically activated AA leucine having the end carboxyl group converted to ethyl ester.[16] Here we encounter the familiar intelligent intervention by OoL scientists necessary to force the desired outcome. AA-esters condense considerably more readily than normal untransformed AAs. In addition, for the condensation to proceed, coordination to Cu^{2+} (copper (II) chloride) was used, and an external pressure of 15 mN⁄m was also necessary, using the mechanical barriers of the Langmuir trough. The final solution was sonicated until a transparent solution resulted.[16]

The surface pressure of 15 mN/m was chosen to orient the surface molecules, facilitating complexing with Cu^{2+} and hindering condensation products from diffusing into the bulk solution. Mechanistically, the Cu^{2+} probably coordinates to the NH_2 of both AAs. Without compression, the adsorbed molecules did not form complexes with the copper ions or react in any way.[16] The authors did not comment on how this affected the relevance to natural conditions.

The condensation was set up to proceed overnight, but the yield of dipeptide was not reported. Whether further subsequent condensation of an AA to a peptide occurs under these conditions was not reported.

We included this example as a representative study, often cited

as evidence, which used conditions known to facilitate peptide condensation. Although an interesting experiment, its quantitative value is highly questionable. Unless one reads the paper carefully, most would not realize, from the references to this work, that instead it reflects the enormous intellectual effort necessary to obtain even the most modest results with no link to natural conditions.

To be relevant to an abiogenesis scenario, one must assume there could have been a constant supply of esterized AAs delivered to this location. No effort was invested to extrapolate from the ideal conditions, which included esterized AAs, ideal surface pressure applied in the correct direction only, sonication, and suitable stoichiometries. The time to build up a relevant concentration of dipeptides and then larger peptides would have been immense, and during this entire time the residues would have been racemizing. Incidentally, we suspect that coordination of Cu^{2+} would have rendered the two amino groups partially positively charged, facilitating formation of the carbanion intermediate and thereby racemization. More Cu^{2+} would facilitate dipeptide formation, but also racemization.

Largest oligomers produced without catalysts

Given the very low yields of oligomeric products reported in OoL studies, in spite of highly contrived conditions as mentioned above, Cronin and colleagues at the University of Glasgow developed a digitally controlled reactor system they called the 'abiotic peptide synthesiser' to run many experiments in parallel to find the best parameters possible.[17] This is valuable work and something we long hoped to do ourselves, demonstrating the best outcome possible under naturalistic conditions, assuming blind chance were to have found these best conditions. Parameters tested included initial concentration of glycine (G, 10^{-4} M – 10^{-1} M), dehydration times (1–96 h), number of dehydration cycles (1–4), temperature (90–130°C), pH (2.15–10), and concentration of NaCl (0–1 M).[17]

The optimal conditions involved injecting an aqueous solution of glycine (0.09 M) containing NaCl (0.25 M), pH ~ 10 adjusted with NaOH, into a preheated vial (130°C), which was then maintained at that temperature for 15 h, evaporating the solution to dryness (the 'dehydration step'). After only one dehydration–hydration cycle, they observed oligomers in solution with sizes up to Gly_{12}.

The pH had a significant influence. For pH 3.5–7.5 at 130°C, the G → oligomer yield was only ~ 1% of that found at pH ~ 10.[18] Unfortunately, the authors did not report what the largest oligomer would be at an OoL realistic pH near neutral. At neutral pH, glycine monomers are zwitterionic, and interactions between the charged amino group and a charged carboxyl group render them ineffective for dimerization. As pointed out above, zwitterions are thermodynamically very stable in water, hindering the condensation reaction.

Raising the temperature from 90°C to 130°C increased the oligomer yield significantly. At 130°C the highest yield (~ 45% at pH 9.75) was achieved in 15 h. Then the yield decreased steadily with time and a brown colour developed (degradation to other substances[19]), as shown in figure 2. At the intermediate temperature of 110°C, the highest yield was reached at ~ 70 h, then decreased also with time. At the lower temperature of 90°C, the monomer → oligomer yield only reached about 20% after 100 h, as shown in figure 2.[20]

Oligomer proportion was highest for NaCl = 0.25 M, but the decrease in oligomer yield at higher and lower NaCl was not dramatic.

More than 4 cycles would not be expected to create higher amounts of the larger oligomers, Table 2 and Figure 3.[21] With each repeated cycle, decomposition of especially the larger oligomers increases. Concentrations of oligomers ≥

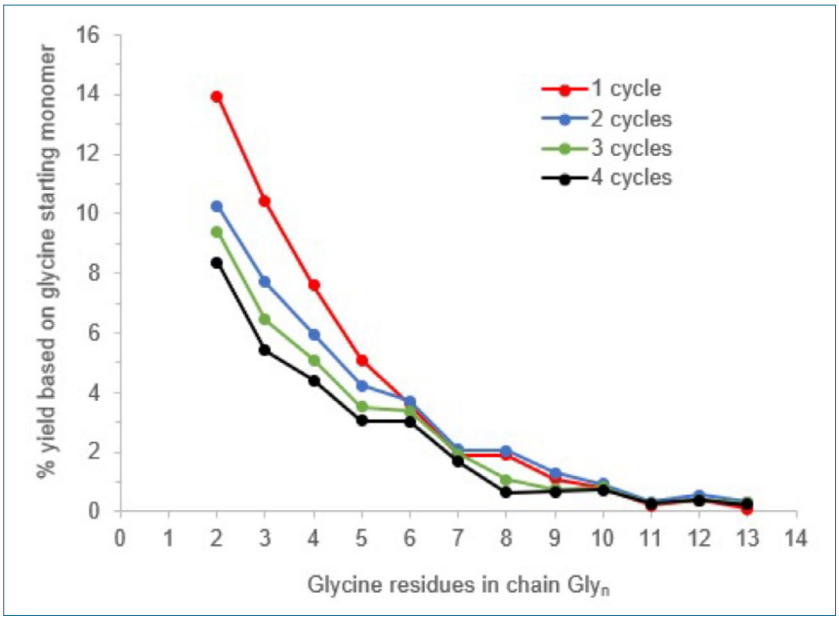

Figure 3. Proportion of glycine oligomers for 1–4 dehydration cycles. Oligomer concentrations are expressed as % of initial glycine. Based on data in table 3 and a figure in the supplementary materials in ref. 17. A pH of 2.61 was used, 130°C, and dehydration time of 24 h.

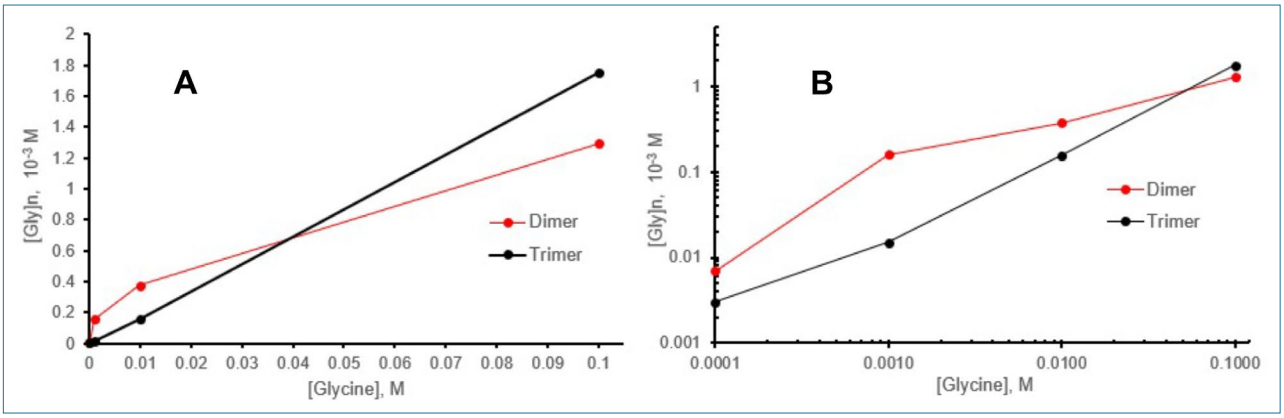

Figure 4. Concentration dependence of Gly_2 and Gly_3 at 130°C.[22] **A**. Plot of Gly vs Gly_2 and Gly_3. **B**. Same data plotted on a log-log scale.

$(Gly)_{14}$ could not be detected by IP-HPLC (ion pair high-performance liquid chromatography).

Larger oligomers like these (13 residues) are only formed when the initial concentration of glycine is very high (and temperature ~ 100°C above room temperature). To illustrate, concentrations of glycine ranging from 10^{-4} to 10^{-1} M were prepared at 130°C. The concentration of the $(Gly)_3$ trimer decreased by almost 3 orders of magnitude when the initial concentration of glycine was decreased from 10^{-1} M to 10^{-4} M, as shown in figure 4B.[22]

A mass spectrometric (MALDI-TOF) analysis revealed a peak at 1181.4, which might be the sodium adduct of $(Gly)_{20}$, the largest oligomer claimed, but at an unmeasurably low concentration.

No attempts were reported to find the largest oligopeptides when different AAs were mixed with glycine. Many AA side chains can react, and indeed both branched structures and linear peptides were found for the four-residue products analyzed.

Interpreting the optimized condensation reactions correctly

All chemists are trained in the same basic principles, and most of us researching in universities and industrial labs have no idea which of our colleagues believe in evolution or creation, since this does not affect our work practices. Most creation-oriented chemists perform the same kinds of systematic experiments Cronin *et al.* did.[17] We often face the challenge of finding optimal manufacturing conditions to produce the highest yield of a target material. Automated equipment to explore parameter space is best practice at big chemical firms. Cronin has done what we always hoped to do someday: find the largest peptides and their concentrations under the best naturalistic conditions possible. We would have favoured a DoE (Design of Experiment) approach, since it is possible that some combination of intermediate values for number of cycles, cycle duration, pH, temperature, and initial concentration of glycine might produce unexpectedly better results. We would have automated the various tests also.[23] Perhaps we could collaborate with the University of Glasgow in an updated project.[24]

But if this had been our project, we would have written a very different paper or research report. We will show that our conclusions are legitimate, having nothing to do with our position on creation vs evolution. We have done operational science for decades and are concerned that Cronin's wish to support evolution has prevented the desired objectivity in communicating his results. The authors are justifiably proud of their results and emphasize that at the best pH, temperature, and dehydration duration, a 0.09 M aqueous solution of glycine monomers produced larger oligomers than others have reported (≤ 7), and in only one cycle. That certainly reflects good laboratory work on their part, and we congratulate them.

But the authors fail to state the obvious. They have intelligently explored all parameters and identified the settings producing the largest Gly_n, knowing this would not occur naturalistically. Gly_{20} may have been obtained, but in too low a concentration to measure. An average-size protein has about 300 residues. Furthermore, they only mention in the supplementary materials that the larger peptides are only sparingly soluble, and the HPLC traces revealed no presence of oligomers of size $n \geq 12$. The solid precipitates which formed had to be dissolved by adding trifluoroacetic acid. As a tarry amorphous material, such peptides would continue to racemize (even fossilized peptides racemize); therefore, they would serve no purpose for abiogenesis speculations.

Since the project was designed for OoL purposes, they should now clearly state that without researcher interference the natural outcomes could only have been far more modest. The question is how much more. That analysis is missing, and this is exactly why we have wanted to perform similar

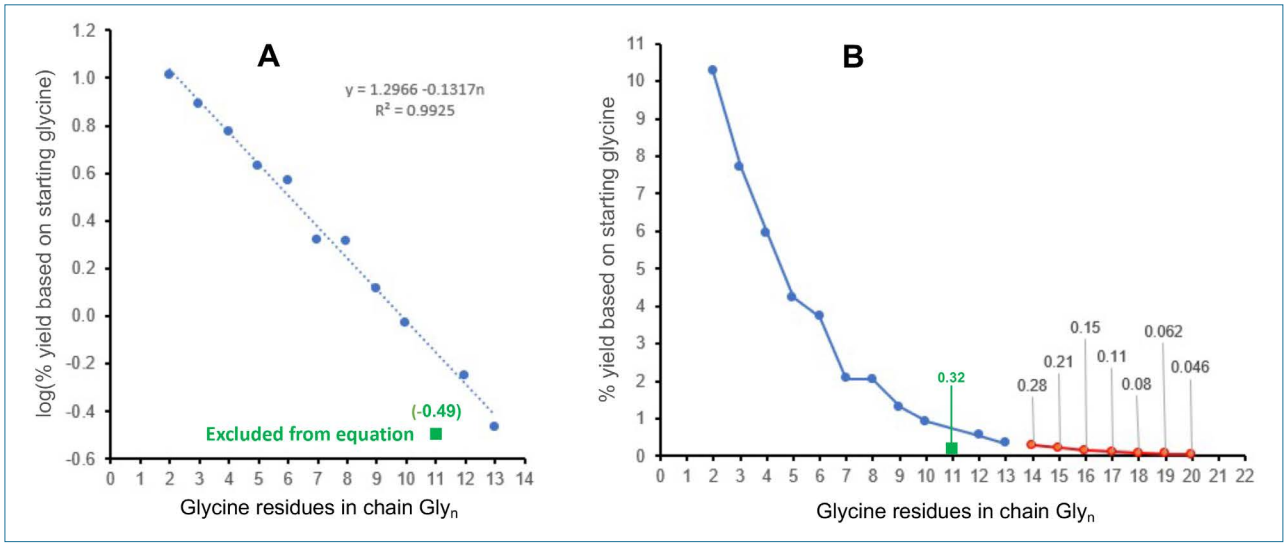

Figure 5. Percent oligomer yields based on starting concentration of glycine, measured at 130ºC after two 24-h hydration–dehydration cycles. **A**. Log (% yield) using data from ref. 21 in blue. An outlier was excluded, shown in green. **B**. Predicted values in red based on the empirical equation shown in A, % yield = $10^{(1.2966-0.1317n)}$, where *n* is the number of residues in peptides Gly_n. The excluded outlier is shown in green.

experiments for years; to extrapolate from theoretical best case towards realistic conditions.

From figure 3 we see that the concentration of peptides decreases rapidly with size. Bada, a leading evolutionist specialist in abiogenesis and amino acids, estimated a concentration of ~ 10^{-10} M would have been available in putative ancient oceans, and not ~ 0.1 M.[10] Cronin *et al.* showed that decreasing the concentration of initial glycine by a factor of 10^3 lowered the concentration of Gly_2 and Gly_3 by a factor of 185 and 583, respectively (figure 4). Not only would this decrease drastically the concentration of the larger peptides over-proportionally, but the diluted dimers and trimers would now rarely come into contact, which was a major reason that larger peptides formed in these experiments. Instead of two now extremely dilute peptides condensing, the polymerization rate becomes almost entirely limited to a peptide having to encounter an amino acid, leading to a peptide only one residue larger. We see once again that deep time is evolutionists' great enemy, as the now much longer times involved provide opportunities for decomposition by exposure to heat and photolysis.

Isolation of water in a hot environment followed by evaporation would increase the density of AAs from an initial ~ 10^{-10} M, but also the concentration of many interfering substances, including those mentioned on part 2 which accelerate racemization. The amino acids could not have been delivered from the sources of heat (volcanos, fierce greenhouse effect, meteorite crashes, or hydrothermal vents), since they would have decomposed. Normal lake shore evaporation would be nowhere near 130°C, and condensation between solidified salts won't occur. A theoretical OoL scenario is not clear. Perhaps the AAs could be delivered by colder water, in which case, after some evaporation, the AAs and peptides would be sure to be diluted again later. But the alternative would be to have them trapped in a hot environment where chemical decomposition becomes inevitable in a matter of hours.

Let us continue modifying the parameters slightly in the direction of more realistic conditions. We will allow for a very heavy dose of optimism in all the parameters, but not to the point of requiring a miracle. The ideal highly basic pH contributed a factor of 10 to 100 improvement. Fifteen hours gave the right results at 130°C. Fifteen hours, not days, millennia, or million years, during which the larger peptides would have completely decomposed. Nature does not work in the manner an astute chemist does. How likely is it that somewhere at around 130°C a collection of concentrated AAs with no interfering substance remained in the right location just long enough before fleeing to safety? Fifteen seconds or minutes would have produced no measurable quantity of large peptides. Everything had to be precisely fine-tuned to obtain good results.

It is common practice to develop mathematical models to perform these kinds of what-if analysis (which is why we would have carried out a DoE first to have the necessary data to develop robust equations). As an example of modelling, one could use reported data to predict the concentration of Gly_{14} to Gly_{20} under specific conditions, as shown in figure 5. (Log transformations, as used in figure 5A, are performed to produce reasonably constant prediction errors over wide ranges of y-values). Since the larger peptides are of major interest the concentrations should be measured multiple

Table 3. Chemical conclusions from the optimal oligomerization experiments

	Key take-home messages
1	At the elevated temperatures which led to the best results, the oligomers decomposed rapidly. Since only a few days would have been necessary for their complete destruction, this would have been inevitable over the course of the putative millions of years. The environment would have been flooded with chemical materials, rather than long, straight-chain peptides.
2	Dehydration cycle time had to be set to a mere number of hours in order to produce larger oligomers.
3	Unrealistically high pH values were necessary to achieve meaningful results. At 130°C, the yield of oligomers at pH 9.8 after 24 hrs was about 10 times higher than that at pH 8.1, the pH of current oceans. Unfortunately, the individual concentrations of $(Gly)_n$ at realistic pH values were not reported.
4	The only proteinogenic amino acid not possessing a chiral carbon, glycine, was used. Biological proteins must be formed from enantiopure L-amino acids to produce the specific structures necessary. Reaction of function groups found in the side chains at these high temperatures would produce a smorgasbord of non-linear peptides.
5	The number of larger peptides decreased steadily along the series 2 cycles → 3 cycles → 4 cycles as degradation increased. Better results will not be obtained with more cycles. However, there is no reason these could not continue to occur naturally, eventually destroying virtually all large peptides.
6	Removing water was necessary, but big, tarry oligomers mixed with other substances would serve no abiogenic purpose.
7	A maximum proportion of $(Gly)_{13}$ / Gly ~ 10^{-3} was only possible after having optimized the parameters. The real-world natural results would be orders of magnitude lower. Oligomers of a size relevant for OoL purposes, > 100 residues, will not be produced naturally.
8	Larger oligomers are poorly soluble. $(Gly)_{12}$ was the largest peptide detectable in aqueous solution by IP-HPLC, and $(Gly)_{14}$ formed a solid precipitate after adding trifluoroacidic acid to render it soluble.

Table 4. Experiments necessary to provide a realistic perspective in ref. 17

	Some suggested experiments to perform
1	Extend our figure 3, which used only 1 to 4 hydration–dehydration cycles, measuring the % yield of each oligomer. (See supplementary materials, table 3 and figure 14 of ref. 16.) Perform six more cycles, since our Table 2 shows a systematic pattern of decrease in peptide concentration when going from two to four cycles. For example, the concentration of Gly^{13} in mM decreases as Cycle 2: 0.34; Cycle 3: 0.30; Cycle 4: 0.25, and we expect that after only about 10 cycles very few peptides of size > 10 residues will survive degradation.
2	Extend reference 16, supplementary materials figure 7, which documented the concentration of oligomers using dehydration durations of only 1, 3, 5, 7, 15, and 24 h. Perform three more experiments, also at 130°C, with durations 48, 96, and 192 h, measuring the concentrations of Gly_{13} and identifying what the decomposition products formed are and their change in concentrations over time. We expect that after only one cycle of duration 192 h very few peptides of size > 10 residues will survive degradation. Note also in ref. 16, supplementary table 1, and figure 5 in the main text of ref. 16, that the total yield of oligomers reached a maximum at 15 h, then steadily decreased with duration of dehydration time.
3	Conduct experiments under the conditions as 2, above, for durations 24 and 192 h, but at temperatures 30, 50, and 70°C. This will show that extrapolating to reasonable temperatures will produce very few of the larger peptides.
4	Use other proteinogenic amino acids, having an alkyl side chain and more complex functional groups and document the concentration of linear oligomer yields, and what alternative products are formed. Reactions involving side chains will hinder the formation of linear peptides and produce many insoluble materials.
5	Document concentration of oligomers for one cycle over a range of dehydration times, beginning with pure alanine instead of glycine. Use a temperature of 130°C and a pH = 9.75, measuring the concentrations of both L and D-enantiomers using HPLC.

times, since small measurement errors would hinder good extrapolations.

We summarize in table 3 some straightforward insights gained from the data provided.[17] Unfortunately, the reader's attention was not drawn to these.

Cronin's pro-evolution bias also seems to be reflected in the fact that several experiments were not conducted which would have been expected from these kinds of agenda-free optimization projects. Conducting such experiments and commenting on them would have offset the distortions perhaps unintentionally provided through the publication.[17] For example, in the supplementary materials, figure 16, an IP-HPLC trace is shown for oligomerization products formed at 130°C and 24 h. The baseline is flat where Gly_{12} and larger peptides were to be found. How reliable are the reported concentrations? But importantly, experiments were run only at the improbable temperatures of 90–130°C.

Why wasn't a single example run at ~ 50°C, with all other parameters set at the optimal values and then the corresponding IP-HPLC shown in order see what size peptides could be obtained, to provide the reader with some perspective to realistic natural outcomes? Seeing a flat baseline starting around perhaps > Gly_8, despite all the other unrealistic parameter settings (glycine 10^8 times higher than plausible, etc.), would present a correct but, for OoL purposes, inimical picture. Table 4 summarizes some of the tests we suggest be performed and evaluated publicly.

We are grateful for Cronin's quantitative work, which confirms that the theoretically largest peptides which could form (~ 20 residues in the case of glycine) would only be in trace concentrations after optimizing every environmental parameter. Peptides formed of size ≥ Gly_{13} would be insoluble. This places severe constraints on the sizes and concentrations of peptides able to form under plausible natural environments and a basis for fruitful discussion on OoL speculations.

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18. Rodriguez-Garcia *et al.,* ref. 17, supplementary materials, figure 12 and table 2
19. Rodriguez-Garcia *et al.,* ref. 17, supplementary materials, figure 10.
20. Rodriguez-Garcia *et al.,* ref. 17, supplementary materials, figure 9 and table 1.
21. Rodriguez-Garcia *et al.,* ref. 17, supplementary materials, table 3.
22. Rodriguez-Garcia *et al.,* ref. 17, supplementary materials, figure 15 and table 4.
23. Instead of varying variables at different settings as done here, where we are employed, we would have first done a mathematical DoE (Design of Experiment) in order to gain the maximum information using fewest experiments. This would have led to other reaction settings than those reported here, to cover interaction effects between variables. This would have provided optimal data to develop mathematical models for extrapolation and interpolation, and to display the effects graphically. Despite the immense amount of valuable data collected, the authors cannot tell us for sure what the best settings would be, nor what values for Gly_n would result within the space of parameters they have explored.
24. If this project were of commercial interest, my employer might have financed this project for Cronin, like many other university research projects we fund.

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